Vol. 47, 1972

Acta Haematologica

International Journal of Haematology
Journal International d Hématologie
Internationale Zeitschrift für Hämatologie

Official Organ of the European Division of the International Society of Haematology



S. Karger · Basel · München · Paris · London · New York · Sydney

Index

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Arnold-Böcklin-Strasse 25, CH-4011 Basel 11 (Switzerland)

Printed in Switzerland by Basler Zeitung AG, Basel

Acta haemat 47 1-12 (1972)

Aregenerative Anemia[1] with Hypercellular Sideroblastic Marrow

A Preleukemic Condition[2]

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Abstract 23 patients with aregenerative anemia from the hematology section at the Karolinska Hospital were followed The patients were divided into 3 groups those with a hypocellular marrow, those with a hyper cellular marrow and those with marrow fibrosis. Most of the patients with hypocellular marrow had granulocytopenia, but no thrombocytopenia, and they did not develop leukemia. The group of patients with hypercellular marrow had no granulocytopenia, but thrombocytopenia and sideroblastosis, and they often developed leukemia. It seems justified to look upon aregenerative anemia with hypercellular marrow and sideroblastosis as a preleukemic state The change in cellular metabolism (sideroblastosis, possible prolongation of generation time) preceded the leukemia by several years but the cellular differentiation appeared normal prior to leukemia development The sideroblastosis may be related to the early changes in iron metabolism previously demonstrated in cancer patients

Key Words
Aregenerative anemia
Preleukemia
Sideroblasts

Aregenerative anemia (a a) is not an entity but a collective term for various disorders having the refractory anemia as a common denominator The etiology of the anemia is often unknown, but some cases develop into leukemia With the present follow up [7] of a a an attempt was made to establish whether any particular type of a.a is likely to develop into leukemia and whether any particular characteristic of the anemia can be used to predict the development of leukemia

[1] Secondary anemias XIV

[2] Supported by the Swedish Cancer Society

To diagnose a fully developed leukemia does not usually offer any particular difficulties, but the recognition of the preleukemic state is often difficult. Diagnosis even of the very early phases of leukemia would be useful for two reasons. First, to begin treatment when there are only few tumour cells present and secondly, to increase our knowledge about the early metabolic disturbances which sometimes seem to cause anemia even before the leukemia becomes manifest.

Material

All patients diagnosed as a.a. in the hematology section during the years 1965–1970 were included in this study. Clinical details for the patients are given in tables I, II and V. In a previous study [7] a.a. was defined as anemia with an erythrocyte radioiron incorporation below 55%. Since radioiron studies were not always done in the present study, the diagnosis of a.a. was also made if, in the absence of a nutritional or hemolytic pathogenesis, the hemoglobin value was below 11 g per 100 ml, or if there was concomitant thrombocytopenia or granulocytopenia and if the condition was refractory to any treatment other than blood transfusions. However, in a subsequent study [13] of the treatment of a.a. with anabolic steroids, it will be shown that not all forms of a.a. are refractory to such steroids. The definition used here will thus have to be revised later.

Blood values, including the latest available hemoglobin values, numbers of red and white cells and differential cell counts, as well as the duration and course of the disease are shown in tables III to V.

Sternal marrow was studied in all patients. For the study of marrow sections pieces of marrow were collected, fixed in acid formalin and embedded in paraffin. The sections were routinely stained with hematoxylin-eosin, and by Hueck's method for demonstration of iron pigment in the reticulum cells of the bone marrow. Smears of bone marrow were stained by the May-Grünwald-Giemsa method for the cytological examination of the cells and when there was demonstrable iron in the sections for the demonstration of iron pigment in the erythroblasts (sideroblasts). Only cells with more than 6 iron granules were counted as sideroblasts. Thus defined, the frequency of sideroblasts ranged between 0 and 14% in a control group of 5 hematologically normal patients.

Results

On the basis of the morphology of the bone marrow sections the patients could be divided into 3 groups. The first group included those with a hypocellular or aplastic, fatty marrow (tables I and III). The

Table 1 Clinical description of patients with hypocellular marrow

Initials	Sex	Age	Hematological diagnosis at autopsy	Clinical description
I W (d)	F	62	apl an	Previously healthy, no positive serologic tests for autoimmune disease, no splenomegaly or hepatomegaly Not seen at Karolinska 1966–70
M C (d)	M	18	apl an	From 7 years of age unspecific allergy treated with antihistamines Final admittance because of purpura and septicemia Finally severe pancytopenia, death in cerebral hemorrhage
R J (d)	F	21	apl an	Previously healthy nurse with rapidly developing aplastic anemia Had been taking iron tablets for Hb 11 3 one month prior to admission The last week infections in the lower jaw Treated with anabolic steroids and corticosteroids and intravenous inoculation of hematopoietic cells from fetal liver without obvious effect Death in a picture of aplastic anemia and Candida septicemia
A A (d)	F	60	apl an	Charwoman with a gastric polyp and anginal pain, treated with nitrites
A E (d)	M	78		Previously healthy widower with aplastic anemia for 3 years Cerebral hemorrhage 6 months prior to death after 2 years treatment of anemia with anabolic steroids and corticosteroids Died of myocardial infarction No autopsy performed
S G (d)	F	60	apl an	Resection of ovaries for ovarian carcinoma 3 years earlier, thereafter treatment with ^{60}Co and alkeran, totally 144 mg No evidence of recurrence of the cancer Hemosiderosis of liver (received about 60 blood transfusions)

(d) = follow up until patient's death

Table II Clinical description of patients with hypercellular marrow

Initials	Sex	Age	Hematological diagnosis at autopsy	Clinical description
R I (d)	F	48	no autopsy	Previously lymphadenitis, salpingitis, herpes zoster and hypothyroidism. Tryptophane load negative. Four to ten weeks of 300 mg/day pyridoxine therapy without effect. Plasma ^{59}Fe half life = 25 min.
L S (d)	F	58	sideroachr a	Previously healthy. No intercurrent disease except diabetes. Erythrocyte incorporation of ^{59}Fe 10%. Approximate erythrocyte life span 50 days. Pathologic tryptophane load test. Episodes of pneumonia and liver damage. Death with meningitis.
E M (d)	F	47	acute blast leukemia	Previously healthy. No intercurrent disease. Death with candidosis and purpura
B B (d)	M	67	acute leukemia	Previously asthma, rheumatoid arthritis and gastric resection. Last seen 2 years after initial anemia, 2 years prior to publication
K W (d)	F	41	ac stemcell leukemia	Previous myocarditis. Granulocytopenia with fever 2 months prior to death. Death in Stevens Johnson like syndrome with erythema multiforme and tonsillary angina.
T E (d)	M	71	promyelocytic leukemia	Gastric resection 18 years earlier because of peptic ulcer. Coombs' direct test slightly positive. Tryptophane load negative. Treatment with iron and folic acid without effect.
A W	M	64	alive	Admitted after 1 year of weight loss, lymphadenites with abscesses and bronchopneumonia later. Gout treated with butazolidin.
A W-M	F	71	alive	Previously healthy housewife with eosinophilia up to 11%. Plasma elimination of ^{198}Au (colloidal) and ^{59}Fe slow. Anemia despite pyridoxin and anabolic steroids.

Table II (continued)

Initials	Sex	Age	Hematological diagnosis at autopsy	Clinical description
B T	F	25	alive	X ray technician with refractory hyposideremia and anemia since age of 7 Previously described [19] No abnormal hemoglobin Normal gynecology Autohemolysis normal with and without glucose Large amounts reticuloendothelial bone marrow iron Sideroblasts 4% [51]Cr curve decreases 11 7% daily (normal less than 2 5%)
S S	M	60	alive	Admitted for moderate thrombocytopenia lasting 1 year causing retinal bleeding
H.W	M	56	alive	Anemia, thrombocytopenia and hematuria (possibly because of glomerulonephritis) Moderate cardiac failure treated with digitalis
R H	M	39	alive	Previously asthmatic social worker admitted for severe anemia with negative serologic tests for autoimmune disease On one occasion after steroid treatment M tuberculosis found in gastric lavage, but no manifest tuberculosis Treated with INH and PAS Slow plasma clearance of radio-iron and radio-gold
I W	M	55	alive	Asthmatic divorcee with cardiomegaly admitted for hemoptysis Anemia refractory to tuberculo-statics and anabolic and corticosteroids
P A	M	46	alive	Previously healthy butcher with negative serologic tests for autoimmune disease, but moderate hypoalbuminemia and hypergammaglobulinemia

(d) = follow up until patient s death

Table III. Blood values in patients with hypercellular marrows

Subject	Hb g/100 ml	RBC, mill	WBC	Differential count bl/poly/mono[2], %	Platelets thousands	Duration of anemia	Sideroblasts %
I.W.	9.7	–	1,800	0/49/51	41	20 months	0[3]
M.C.	5.1	1.7	2,100	0/42/58	6	2 months	0[4]
R.J.[1]	12.6	–	250	0/0/90	4	2 months	0[4]
A.A.[1]	9.0	3.1	1,700	0/30/70	12	2 months	10
A.E.	9.0	2.9	5,500	0/72/28	224	3 years	8
S.G.	9.0	–	3,200	0/37/63	144	4.5 years	0[3]

[1] Transfusion within last 3 weeks
[2] bl = blast cells, poly = polymorphonuclear leucocytes, mono = monocytes
[3] Too few erythroblasts to count
[4] No stainable iron in marrow section

Table IV. Blood values in patients with hypercellular marrows

Subject	Hb g/ml	RBC, mill	WBC	Differential count bl/poly/mono	Platelets thousands	Duration of anemia	Sideroblasts %
R.F.	7.8	2.0	3,000	2/59/39	216	9 years	70
E.S.	8.3	–	9,500	0/80/20	95	7 years	[1]
E.M.	7.2	–	450	4/6/90	9	8 years	0[2]
B.B.	7.3	2.7	109,000	20/36/44	161	2 years	10
K.W.	9.6	2.9	2,900	0/1/99	233	2 months	0[2]
E.E.	9.3	–	2,800	0/65/35	22	2 years 7 m	45
A.W.	10.3	3.4	9,200	0/36/64	96	7 months	14
A.W.M.	10.0	–	2,500	0/52/48	214	5 years	55
B.T.	11.3	–	3,100	0/56/44	168	20 years	2
S.S.	14.9	4.3	8,000	0/49/51	93	2 years	32
H.W.	6.4	–	4,100	2/64/34	10	4 years	16
R.H.	10.9	3.5	59,000	22/56/22	44	15 months	35
F.W.	5.5	1.8	1,700	2/13/85	23	2 years	85
E.A.	8.4	2.5	2,200	0/14/86	45	7 years	0[3]

[1] Probably high percentage but unsatisfactory smear
[2] No stainable iron in marrow section
[3] No smear available for sideroblast stain

Table V Patients with marrow fibrosis

Initials	Sex	Age	Hb	Blood values r	Blood values w	Differential count bl poly/mono	Trb	Duration of anemia	Sideroblasts, %
S W	F	44	9 1	–	1,700	0 49/52	124	16 months	0
G N	F	36	9 2	2 7	24 000	23 69 8	798	8 years	0
S A (d)	M	52	10 4[1]	–	1 800	0 49/51	18	8 years	18

(d) = follow-up until patient s death

[1] Transfusion 3 weeks prior to sample

S W Previously encephalitis, hydrocephalus, possible pituitary cachexia (weight 35 kg, total *body potassium 1 39 g/kg body weight*), and healed pulmonary tbc Several positive Wasserman reactions.

G N *Saleslady with a history of splenomegaly and possible hemolysis 2 years prior to admission* An increased blood volume and terminal postsplenectomy thrombocytosis may suggest previous myeloproliferative disease

S A Previously healthy clerk with normal Schilling test, FIGLU test, reticulocytes, but long standing pancytopenia, despite corticosteroids, anabolic-steroids Moderate hemolysis (29 mg hapto-globin 100 ml) Later coronary disease and death at home No autopsy performed

second group included patients with a hypercellular marrow with little fat (table II and IV) and the third group of patients had marrow fibrosis (table V)

Patients with hypocellular marrow Five of the patients with hypocellular or aplastic marrow could be followed to autopsy, and in each case the autopsy findings were consistent with aplastic anemia In 4 of the 6 patients no sideroblasts could be found in the marrow In other 2 patients the number of sideroblasts was within the normal range, and thus none had sideroblastosis In none of these patients was there any indication of a progress of the disease into leukemia

The duration of the disease ranged between 2 months and 4½ years All except one of these patients had granulocytopenia, and 4 of the 6 had thrombocytopenia

Patients with hypercellular marrow Six of the 14 patients with hypercellular marrow have died (table II) Four of these died in acute myeloblastic leukemia or promyelocytic leukemia, and in only one patient was the diagnosis at autopsy aregenerative anemia No autopsy was performed on the sixth patient.

Eight patients are still alive. In patient R.H. the white cells are markedly increased and he has 22% blast cells in his differential cell count (table IV). For this reason this case may be considered to be in an early phase of blast leukemia.

The bone marrow had been examined in all these cases before the overt development of leukemia. There was very active erythropoiesis and granulopoiesis, but normal differentiation and no clearly atypical cells. Moderate amounts of iron pigment could be demonstrated in the reticuloendothelial cells, except in 2 patients who presumably did not have an increased sideroblast number. For 2 patients, no smears were available for sideroblast stains. Eight of the 10 patients examined in this respect had an increased sideroblast count, unlike the low or normal number in the patients with hypocellular marrow. In only 2 patients were the sideroblast counts equivalent to those in the control patients.

Thrombocytopenia of varying degrees occurred in 9 of the 14 patients with hypercellular marrow. Only one of them had granulocytopenia, as compared to almost all of the patients with a hypocellular marrow.

The duration of the disease was considerably longer in this group than in the group of patients with hypocellular marrow. Only one patient died within a year, and most patients have survived for several years, up to 9 years, after the diagnosis of a.a. with hypercellular marrow was made.

Patients with marrow fibrosis. Of the 3 patients diagnosed as having marrow fibrosis with aregenerative anemia one died 8 years after the onset of the disease (table V) and he had pancytopenia. The duration of the disease in the other 2 cases was 16 months and 8 years; they had leukocytosis and thrombocytosis.

No iron was found in the marrow sections in 2 of the cases, whereas slightly elevated sideroblast values were encountered in the patient that died.

Discussion

Previous results. In previous studies preleukemic stages have been described preceding approximately 5–10% of acute leukemias [25, 30] and in about 20% of patients with sideroblastic anemias [28]. Preleukemia is sometimes characterized by a lack of alkaline phosphatase in neutrophils [25], by Pelger-like cells [22, 25], by sideroblasts [5, 8, 16], by PAS-positive erythroblasts [8], by megaloblastosis and/or by hyperseg-

mentation [8, 22], erythroblastopenia [24], leukopenia [17], and pancytopenia [3]

Possible preleukemic marrow aplasia [4, 15, 31] has been described as well as preleukemic hyperplasia [15, 22] However, the comments regarding marrow cellularity may be tentative in some of the previous studies since bone marrow sections were not studied in all these cases

Chromosomal abnormalities have been described preceding some leukemias [14, 23, 27] In some patients, a history of allergy was found [6] as was the case in patients with agranulocytosis [12] In other studies [8] abnormal erythrocyte antigens were found

None of these signs has yet been considered to be sufficiently pathognomonic [22] to warrant cytostatic treatment. Nor has immunotherapy been described in this stage of leukemia Treatment with pyridoxine [12] or prednisone [12, 25] in a few cases was ineffective

Since it has been claimed that testosterone may inhibit spontaneous leukemia [28], a systematic study of anabolic steroids is in preparation at present [13] One earlier study [12] unfortunately did not show particularly promising results Some cases with hypercellular marrows under treatment appear to show some improvement, but less frequently than the cases with hypocellular marrow

Present study The term 'aregenerative anemia' is used as a clinical term in this article, irrespective of its etiology That a hypocellular or aplastic marrow can be followed by aregenerative anemia is easily understood, although the cause of the marrow failure is usually unknown That a hypercellular, histologically and cytologically hyperactive marrow can be accompanied by aregenerative anemia as shown by the present material is more difficult to explain Somewhat similar refractory anemias occur in patients with cancer [2, 20], and are accompanied by a disturbed iron metabolism It is uncertain, in fact, whether the 2 forms of refractory anemia are really similar, but both in the secondary anemia in cancer, and in the refractory anemia preceding leukemia moderate hemolysis and decreased ^{59}Fe incorporation in erythrocytes can be found [2, 7, 11, 20]

The decreased ^{59}Fe incorporation and the only moderately decreased red cell life span suggest, in spite of the histological and cytological indications of increased erythropoiesis and granulopoiesis, that the mean generation times may actually be prolonged [1] The cause of this possible change is quite unknown, since no atypias were found and the differentiation appeared morphologically normal before leukemia developed

One abnormality was encountered, however. A very common finding was an increase in the number of sideroblasts in the hypercellular marrows. In some cases the increase was considerable. Further studies are in progress to examine this sometimes apparently preleukemic deviation from the normal cellular metabolism. Even at present, however, a determination of the number of sideroblasts may well be a valuable diagnostic and prognostic sign.

There are indications, as mentioned above, that the hypercellularity of the marrow can reflect profound alterations in cellular proliferation which cannot, however, be demonstrated cytologically. Almost all patients who died in the group with hypercellular marrows died of leukemia. If the possible prolongation of generation times in bone marrow cells found in leukemia [18, 26] precedes the manifest leukemia by several years, while maturation is still normal, a preleukemic form of aregenerative anemia would be seen. Later, when maturation also becomes disturbed, a leukemic picture could develop. In addition to the probably prolonged generation times, there is another demonstrable change in cellular metabolism preceding the leukemia by several years, namely the sideroblastosis. This may be relevant in connection with the early changes in iron metabolism in cancer patients [11].

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Acta haemat. 47: 13–20 (1972)

Treatment of Aplastic Anaemia with Methenolone

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Abstract. Treatment of aplastic anaemia in 28 patients with oral methenolone 1–2 mg/kg for from 2 to 12 months resulted in normalisation of the blood count in 12 cases (43%). The remission rate was 60% for patients with a treatment period of at least 3 months. Cholestatic jaundice occurred in 2 cases (7%) but these recovered after discontinuing the drug. Other side effects were not severe enough to cause interruption of the treatment. There seems to be no doubt that methenolone by mouth is a useful treatment of patients with aplastic anaemia. The trial of therapy should be extended to at least 6 months.

Key Words
Anabolic hormones
Aplastic anaemia
Bone marrow failure
Methenolone therapy

The introduction of testosterone in the treatment of aplastic anaemia in children [9] was followed by less successful trials in adult patients [4, 8, 10]. Later on, anabolic steroids with less androgenic activity were used in place of testosterone and good results were achieved in both children [1, 3, 5, 6] and adults [2, 5, 6, 11]. This paper reports our results in the treatment of patients with aplastic anaemia with methenolone.

Patients and Methods

The series of patients consisted of 28 patients with aplastic anaemia and other refractory anaemias, treated in the Department of Medicine, University of Oulu, the Fourth Department of Medicine, University of Helsinki, and in Kivelä Hospital, Helsinki, during the period from May 1969 to February 1971. The results were evaluated up to May 1971. Most patients had normochromic anaemia that had been refractory in earlier treatment. Some patients with a history of the use of drugs known to cause aplastic anaemia had selective neutropenia or thrombocy

Table I Clinical and haematological

Case No	Age years	Sex	Aetiology	Previous duration of the disease	Previous treatment	Need of trans-fusions units/month
1	38	M	idiopathic	5 years	prednisolone, testosterone, oxymetholone	–
2	21	M	idiopathic	–	–	–
3	19	F	idiopathic	–	–	2–4
4	63	F	phenylbutazone, sulphonamide	2 months	prednisolone	4
5	64	F	phenylbutazone, gold	2 years	prednisolone	–
6	61	M	myelocytic leukaemia, busulphan	1 year	prednisolone	2
7	50	M	myelocytic leukaemia, busulphan	5 months	prednisolone	–
8	25	M	idiopathic	2 years	prednisolone	–
9	58	F	myelomatosis, no cytostatic agents	5 years	prednisolone, testosterone	3
10	50	F	myelofibrosis	–	–	1–2
11	51	M	idiopathic	7 months	prenisone, testosterone	1–2
12	28	F	chloramphenicol	5 years	prednisolone, testosterone	–
13	80	F	myelomatosis, melphalan	3 years	–	1
14	58	F	idiopathic	–	–	1–2
15	20	M	idiopathic	10 years	testosterone	–
16	4	M	idiopathic	4 months	prednisone	1–2
17	61	F	idiopathic	1 year	–	3–4
18	55	F	idiopathic	4 years	–	4–6
19	65	F	idiopathic	2 years	prednisone	2
20	54	F	idiopathic	1 year	testosterone	4
21	65	F	sulphonamide	2 months	prednisolone	–
22	53	M	myelofibrosis	3 months	–	–
23	48	F	myelofibrosis	–	–	–
24	62	M	myelofibrosis	–	–	–
25	41	M	idiopathic	2 months	prednisolone	–
26	77	M	myelomatosis, no cytostatic agents	2 years	–	–
27	67	M	myelomatosis, no cytostatic agents	1 year	prednisolone	2
28	70	M	idiopathic	5 years	prednisolone	2

data of the 28 patients in the trial

Blood count before treatment			Dosage and duration of treatment	Blood count after treatment			Result
Hb, g/l	neutrophils $\times 10^9$/l	platelets $\times 10^9$/l		Hb, g/l	neutrophils $\times 10^9$/l	platelets $\times 10^9$/l	
93	0 7	50	100 mg *per os*, 9 months	120	3 0	75	remission
108	1 0	60	100 mg *per os*, 5 months	127	2.3	80	remission
44	0 8	100	100 mg *per os*, 10 months	157	9 0	140	remission
54	0 24	30	100 mg *per os*, 6 months	129	1 3	100	remission
160	2.5	50	100 mg *per os*, 1 month	137	3 7	150	remission (I)
152	6 0	36	100 mg *per os*, 5 months	151	5 0	260	remission (II)
76	1 3	25	100 mg *per os*, 5 months	141	3 9	110	remission
98	1 0	14	50 mg *per os*, 3 months	132	2.0	70	remission
110	2 0	36	50 mg *per os*, 2 months	126	4 0	110	remission
60			100 mg i m /week, 1 year	143	4 3	224	remission
59	3 3	20	100 mg i m ×2/month, 20 months	138	3 8	208	remission
67	0 34	60	150 mg *per os*, 17 months	139	2 2	50	remission
80	6 0	62	100 mg *per os*, 2 months	123	9 0	95	remission, jaundice
90	3 9	191	100 mg i m ×2/month, 1 year	100	3 5	165	improvement
54	1 8	209	150 mg *per os*, 6 months	84	1 4		improvement
72	1 7	42	150 mg *per os*, 5 months	98	1 3	27	improvement
99	0 09	2	50 mg *per os*, 2 months	108	0 9	18	improvement
66	1 7	12	200 mg *per os*, 6 months	59	0 9	15	failure, died
56	2.0	92	150 mg *per os*, 4 months				failure
90	1 4	55	150 mg *per os*, 2.5 months				failure, died
63	0 09	48	150 mg *per os*, 1 month	75	4 8	50	failure, died
110	0 7	240	100 mg *per os*, 1 month		4 0		jaundice, failure
106	1.2	120	100 mg *per os*, 3 months				failure
126	4 3	12	50 mg *per os*, 3 months				failure
105	12.0	200	50 mg *per os*, 2 months				failure
145	7 0	50	50 mg *per os*, 2 months				failure
83	1 7	150	50 mg *per os*, 2 months	82	1 7	170	failure
72	0 7	18	100 mg *per os*, 1 month	49	0 8	20	failure
68	0 5	30	150 mg *per os*, 1 month				failure

topenia The bone marrow was hypocellular at least at some phase in the course of the disease in about 75% of the patients, in the remaining ones it was normocellular all the time Special attention was paid to the numbers of megakaryocytes in the evaluation of the bone marrows Four patients with refractory anaemia caused by myelomatosis or by cytostatic therapy given because of this condition were included in the series, as well as 2 patients with aplastic anaemia caused by busulphan treatment for myelocytic leukaemia, and 4 cases of myelofibrosis The age and sex of the patients, the aetiology of the condition, the duration of the dis ease from diagnosis to methenolone therapy and previous treatment are given in table I The need for blood transfusions is indicated in this table, and in the pre treatment blood counts the haemoglobin levels may be in part corrected in some cases

The treatment consisted of methenolone orally 1–2 mg/kg body weight (Primo bolan®, Leiras/Schering) Five patients, however, received at least a part of their course as weekly i m injections of methenolone The dosage and duration of the treatment in each patient before assessment of the result are indicated also in ta ble I

The post treatment blood counts in table I are in most cases mean values for 2 or more blood counts thus reflecting the level achieved Blood counts *after trans fusions* were not taken into account

The result was evaluated as a remission if the blood count was normalised (Hb $>$120 g/l, neutrophils $>$1 5$\times$10^9/l, platelets $>$100$\times$10^9/l) However, also cases with sub normal platelet counts (50–100$\times$10^9/l) were included in this group, if no bleeding tendency was noted and no other symptoms were present after the treat ment The result was regarded as improvement if there was no longer need for blood transfusions although the blood count was still sub normal One patient whose blood count was maintained at sub normal level during methenolone but deteriorated after the treatment ended was included in this category (case No 15) The result was regarded as a failure if no definite improvement in the blood count was achieved

Results

A full normalisation of the blood count was achieved in 7 patients In addition, the haemoglobin level and neutrophil count were normalised in 5 other patients whose platelet counts were sub-normal at the time of assessment of the result Thus a remission could be said to have been achieved in 12 patients

In 4 patients an improvement was observed Their blood counts remained sub-normal but the bleeding tendency ceased and there was no need for further transfusions

No effect was seen in 11 patients and of these 3 died However, methenolone treatment of at least 3 months duration had been given in only 4 patients in this group in which no success was observed The du-

gun after withdrawal of prednisolone in March 1970 Over a month the platelet count rose from 50 to 150×10⁹/l (double observations) and she became asymptomatic Because she felt healthy she wanted to discontinue the medication At the end of August she returned with haematomata and a platelet count of 14×10⁹/l A new course of methenolone 100 mg daily normalised the platelet count to 250×10⁹/l in 3 months She is still taking a small maintenance dose of 10 mg of methenolone daily and has remained asymptomatic

Case No 6 A 61 year old banker had suffered from myelocytic leukaemia for 2 years and had been treated with busulphan During the previous 6 months he had received no cytostatic therapy, only prednisolone 10–20 mg daily, and monthly transfusions or anaemia With a haemoglobin level of 70–80 g/l, leukocytes at about 3 0×10⁹/l and platelets at 20–25×10⁹/l, methenolone at 100 mg daily *per os* was begun Five months later the haemoglobin was 141 g/l, leukocytes 5 8×10⁹/l with a normal differential count and platelets 110×10⁹/l His general condition is better than at any time during the previous 3 years

Discussion

Our results confirm the usefulness of methenolone in the treatment of aplastic anaemia [5, 11] The remission rate of 60% for patients treated for a period of at least 3 months is within the range of the reports on the results of treatment with oxymetholone [5, 6], dromostanole [5] and nandrolone decanoate [2] ZITTOUN *et al* [11] reported favourable results in 50% of their 63 cases of aplastic and various refractory anaemias SANCHEZ-MEDAL *et al* [5] in their trial of anabolic androgenic steroids in the treatment of aplastic anaemia gave methenolone to 17 patients but they do not give the data for this group separately from the overall results In their discussion *they* only say that methenolone shares the therapeutic activity of oxymetholone, which was the principal drug in their trial [5]

The sequence of the remission signs in our series was similar to that described in earlier reports [5, 10] The recovery of the platelet count was most delayed and is still incomplete in many cases As we could obtain remissions in some patients even after 5–6 months of initially unsuccessful treatment, we think that the trial of methenolone treatment for aplastic anaemia should be extended to at least 6 months unless remissions or severe complications occur earlier, before the treatment is considered a failure In the light of the present series it seems likely that the remission rate in aplastic anaemia can be improved to up to 70–80% with sufficiently long courses of methenolone treatment at an adequate dosage, 1–2 mg/kg body weight for at least 6 months

The superiority of methenolone to testosterone in the treatment of aplastic anaemia is apparent by the higher rate of remissions, 50–70% [6, 11] against 5–40% [4, 8, 10] in adult series. In addition, 3 patients in our series who had not responded to adequate trials with testosterone during the earlier course of their disease developed remissions during methenolone treatment.

Many of our patients received prednisolone or other corticosteroids during their methenolone treatment. It is difficult to assess the influence of corticosteroids on the results, but it cannot be substantial. Many of the patients had received corticosteroids earlier without effect, and the reports on the treatment of aplastic anaemia with corticosteroids show that their effect on the disease is doubtful [4, 7].

Transient cholestatic jaundice in 2 patients ([illegible]%) was the severest complication in our trial. When treating a disease with a usually fatal course like aplastic anaemia [2, 4, 6, 7, 10], minor side effects like menstrual disturbances, hoarsening of the voice, slight hirsutism and acne are relatively harmless, and none of our patients regarded them as so disturbing that they preferred to discontinue treatment. When comparing these side effects with similar complications during testosterone therapy they are, according to our experience, markedly milder during methenolone.

Acknowledgement. This study was supported by a grant from the Sigrid Jusélius Foundation. Methenolone 5-mg tablets were kindly supplied by the Pharmaceutical Factory Leiras, Turku, Finland.

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Acta haemat 47 21-32 (1972)

Cell Proliferation in the 'Preleukaemic' Phase of Acute Leukaemia

A Cytophotometric and Autoradiographic Study[1]

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Abstract In a 13-year-old patient with acute leukaemia the leukaemic blast cells the nucleated red cells and the megakaryocytes were studied by cytophotometric determination of the DNA content and autoradiographic labelling with ^{3}H TdR *in vitro* In the preleukaemic phase a striking proliferation defect in the early polychromatic erythroblasts was observed consisting of an accumulation of cells in G_1 and a decreased proportion of cells in S In the megakaryocytes low DNA values from 2c-8c as compared to 4c-32c in normal megakaryocytopoiesis, and a decreased number of labelled cells between the ploidy stages was observed indicating a severely restricted polyploidization capacity These results suggest that in the preleukaemic stage a basic proliferation defect of acute leukaemic is becoming apparent in the so-called non leukaemic cell systems leading to peripheral mono- or pancytopenia

Key Words
Autoradiography
Cytophotometry
DNA synthesis
Erythropoiesis
Megakaryocytopoiesis
Preleukaemic state

At the time of the first clinical manifestations of acute leukaemia in most cases an advanced infiltration of leukaemic blast cells (LBC) in the bone marrow and peripheral blood is observed In some patients, however, a variety of haematological abnormalities may be present some time before the diagnosis of acute leukaemia is made The term 'smouldering acute leukaemia' was used to describe such a variant of acute leukaemia, which is characterized by an insidious onset, fatigue for months to years, moderate anaemia, thrombocytopenia and/or leukopenia, and no remarkable physical findings The bone marrow may be hypo-, nor-

[1] Supported by Deutsche Forschungsgemeinschaft

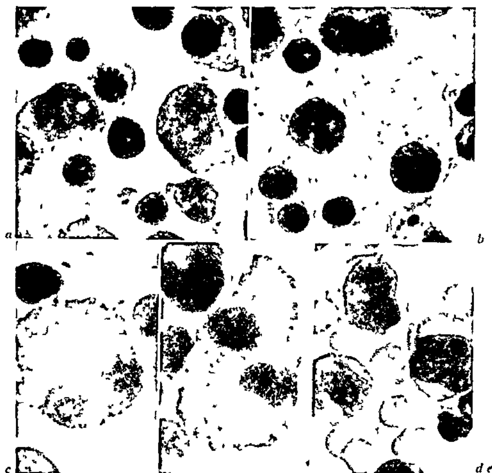


Fig. 1. Single LBC *(a)* and atypical megakaryocytes *(b, c, d)* in June 1969. LBC in the peripheral blood *(e)* in June 1970.

mo- or hypercellular, showing a varying number of LBC from 5–10% to almost complete infiltration [9, 20]. In contrast, in 'preleukaemic states' showing similar clinical symptomatology and duration of months to years, LBC are not present until the onset of the leukaemic phase [2, 21]. Clinically it may be difficult to suspect whether an acute leukaemia will develop or not, and the diagnosis of 'preleukaemic states' can usually be made only in retrospect.

Recently a patient has been observed in our hospital, in which the first clinical symptomatology resembled a severe refractory anaemia. The bone marrow showed erythroblastic hyperplasia, but minimal infiltration of atypical cells which retrospectively were designated as LBC

Table 1 Main haematological data and the results of the bone marrow differentiation

	June 1969	July 1969	Aug 1969	Sept 1969	Okt 1969	Nov 1969	Dec 1969	Jan 1970	Febr 1970	March 1970	April 1970	May 1970	June 1970
Hb g/100 ml		9.9	12.1	6.2	6.9	6.1	7.2	5.4	4.2	11.9	7.1		7.3
RBC 10^6/ml		2.83	4.10	2.01	1.85	2.00	2.32	1.50	1.83	3.98	2.42		
HK %		27		19	17	21	20		19	30	21	20	22
MCH pg		35	30	30	37	31	31	36	23	36	29		
MCV μm^3		95		95	91	105	86		104	75	87		
Reticulocytes ‰		25		4	8	3		3			1	6	2
WBC/mm^3		5 700	3 400	4 300	3 900	3 700	4 400	2 500	4 400	7 000	8 900	188 000	3 300
LBC %		4	4	1	0		2	5			21	54	90
Platelets/mm^3		395 000		532 000	599 000	425 000		235 000	180 000	396 000	357 000	128 000	6 000
Erythropoiesis, *total* %	39.4	49.2		28.8					6.2		4.2	2.2	30.8
Granulopoiesis total %	23.0	16.4		21.2					19.6		13.8	12.8	3.8
E:G ratio	1.71:1	3.00:1		1.36:1					0.32:1		0.30:1	0.17:1	8.11:1
LBC, %	8.6	6.4		21.4					41.6		73.4	77.6	56.4
Lymphocytes, Monocytes, Plasma cells %	29.0	28.0		28.6					32.6		8.6	7.4	9.0

E:G = Erythro-granulopoietic ratio (normal range 0.2:1–0.8:1)

In the following 8 months the proportion of LBC arose slowely to almost complete infiltration The patient died one year after the onset of the first clinical manifestations In this case the proliferation of LBC, of nucleated red cells and megakarycocytes has been investigated by cytophotometric determination of the DNA content and autoradiographic labelling with tritiated thymidine (^{3}H-TdR) *in vitro* at different times during the progress of the disease The results provide evidence of a severe proliferation defect in the non-leukaemic cell lineages, which becomes apparent prior to advanced leukaemic bone marrow infiltration

Case Report

The 13 year old boy was admitted in July, 1969 with a history of headache, fatigue and gastro intestinal symptoms which began two months ago The physical examination revealed no pathological findings except a mild brownish skin colora tion and pale mucous membranes Liver, spleen and lymph nodes were not en larged Laboratory investigations at this time showed a normocytic anaemia The other haematological data were found within normal limits (table I) Bone marrow examination revealed a striking erythroblastic hyperplasia and a maturation arrest of the granulopoietic cell line A low number of atypical cells was observed, showing a large nucleus with a fine chromatin structure and prominent nucleoli, and a pale bluish cytoplasm without granulation The megakaryocytes showed 1–8 relatively small round or oval separate nuclei and a wide and slightly basophilic or neutrophilic cytoplasm with distinct azurophilic granulation (fig 1)

Continuous clinical and haematological controls during the following 8 months did not reveal any new aspects The patient had to be transfused repeatedly In January, 1970, disseminated ekcematic skin lesions developed Skin biopsy showed a mild perivascular inflammatory reaction and no leukaemic infiltration In February, 1970, the proportion of LCB within the bone marrow had increased up to 42% In April a continuous rise of the white blood cells was observed Administration of vincristine, daunorubidomycine and prednisone led to a decrease of the peripheral LBC, but not to remission Further treatment with asparaginase and cytosine-arabinoside was unsuccessful too The patient died in June, 1970 At autopsy, beside leukaemic infiltration of bone marrow liver, spleen and kidney tumorous fungoid infiltrations due to cryptococcus sepsis, and multiple haemorrhages were found

Method

Bone marrow aspirations were performed repeatedly from June 1969, until June 1970 (times designated I–VI in text and figures) The marrow was aspirated into a syringe containing 0.5 ml EDTA solution (1% Na_2EDTA in 0.7% NaCl) For the cytophotometric determination of the DNA content, smears were made from the marrow spicules and stained with the May Grunwald Giemsa stain The individual cell areas were marked with an object marker (Leitz, Germany) and

Table II Technical details of the cytophotometric (C) and autoradiographic (A) study

	No of investigation					
	I	II	III	IV	V	VI
Time	June, 1969	July, 1969	Febr, 1970	April, 1970	May, 1970	June, 1970
Method	C	C	C+A	C	C	C+A
Cell types						
Leukemic blast cells	+	+	+	+	+	+
Erythroblasts	+		+			
Megakaryocytes			+			
Diploid standard evaluated by method[1]	2	2	1 3	2	2	3

[1] Explanation of the methods 1, 2 and 3 see text

single cells were photographed for subsequent localization Thereafter, Pappenheim stain was leached out by treatment with 50% ethanol, and the smears were restained by the Feulgen method applying pararosanilin for Schiff's reagent For the cytophotometric measurements a MPV cytophotometer (Leitz, Germany) was used The nuclear size was determined by planimetry in LBC and megakaryocytes *and by evaluation of the nuclear diameter in the erythroblasts*

For the combined cytophotometric and autoradiographic investigation, the marrow in EDTA was incubated for one hour with ^3H TdR (concentration 2 0 μCi/ml, specific activity 2 Ci/mM) at room temperature After completion of the cytophotometric measurements autoradiograms of the Feulgen stained smears were made by the *dipping film technique The technical details of the combined method* have been described previously [18, 19]

The bone marrow has been investigated at 6 different times (I–VI) The method used and the cells which have been studied at each time are given in table II The classification of the LBC and megakaryocytes was performed in panoptic stain according to the above mentioned cytological criteria *In the erythropoietic* cell line the following cell types were distinguished (1) basophilic erythroblasts (E_{1-3}), (2) early polychromatic erythroblasts (E_4), and (3) late polychromatic erythroblasts (E_5) Apart from the cytoplasmic staining properties cells showing a nuclear area smaller than 20 μm^2 were designated as E_5

Results

Evaluation of the diploid standard For evaluation of the diploid standard (2c) 3 methods are available (1) A *reference* system *of diploid*

In the following 8 months the proportion of LBC arose slowly to almost complete infiltration The patient died one year after the onset of the first clinical manifestations In this case the proliferation of LBC, of nucleated red cells and megakaryocytes has been investigated by cytophotometric determination of the DNA content and autoradiographic labelling with tritiated thymidine (^{3}H-TdR) *in vitro* at different times during the progress of the disease The results provide evidence of a severe proliferation defect in the non-leukaemic cell lineages, which becomes apparent prior to advanced leukaemic bone marrow infiltration

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Cell types						
Leukemic blast cells	+	+	+	+	+	+
Erythroblasts	+		+			
Megakaryocytes			+			
Diploid standard evaluated by method[1]	2	2	1, 3	2	2	3

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Results

Evaluation of the diploid standard. For evaluation of the diploid standard (2c) 3 methods are available. (1) A reference system of diploid

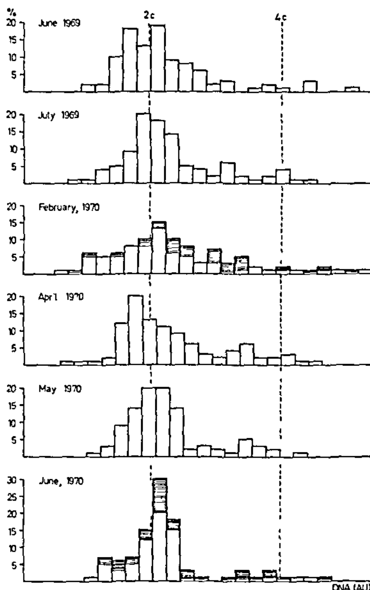


Fig 2 Relative DNA content in arbitrary units (AU) and ³H TdR labelling (shaded areas) of the leukaemic blast cells (LCB) at different times of observation Semilogarithmic scale 2c = mean tetraploid DNA content

cells (e g lymphocytes) is used, and the DNA values of about 50 cells are averaged (2) Within a proliferating cell population showing a high proportion of diploid cells, the DNA values of those cells obviously being diploid are averaged (3) By combined application of the cytopho-

tometric determination of the DNA content and autoradiographic labelling with ^{3}H-TdR *in vitro* the different stages of the cell cycle as G_1 (diploid and unlabelled cells), S (^{3}H TdR labelled cells) and G_2 (tetraploid and unlabelled cells) can be distinguished By this method 2c can be determined by the average of all cells which are in G_1 [19] In table II the method is given which has been used at each time of investigation

Leukaemic blast cells (LBC) The results of the study of the LBC are given in figure 2 The DNA values are distributed from 2c–4c and are showing a striking accumulation at 2c (78–87%) The combined application of cytophotometry and autoradiography at time III reveals 62% in G_1, 29% in S and 9% in G_2 At time VI 61% of LBC are in G_1, 36% in S and 3% in G_2 indicating no significant changes of the distribution of LBC in the different stages of the cell cycle during the progress of the disease

Erythropoietic cells The distribution of the DNA content of the erythropoietic cells at time I is given in figure 3 In E_{1-3} and in E_4 diploid, hyperdiploid and tetraploid cells are observed In E_4 a striking accumulation of diploid cells (69%) and consequently a decreased proportion of hyperdiploid and tetraploid cells is found The DNA content of E_5 is slightly lower than the diploid standard used

At time III (fig 4) a similar distribution of the DNA content in the erythropoietic cells is present The results of the combined cytophotome-

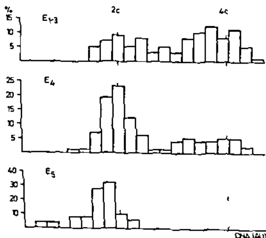


Fig 3 Relative DNA content (AU) of erythroblasts in June 1969

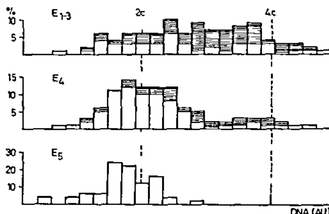


Fig 4 Relative DNA content (AU) and ³H TdR labelling shaded areas) of erythroblasts in February, 1970

tric and autoradiographic study of E_{1-3} reveal 28% in G_1, 57% in S and 15% in G_2. In E_4 67% are in G_1, 25% in S and 8% in G_2. The diploid E_5 cells are competely unlabelled

Megakaryocytes The results of the study of the megakaryocytes are given in figure 5 In the upper graph (a) of this figure, the DNA content of the single nuclei and in the lower graph (b) the DNA content of the whole cell is represented As seen in this figure, most of the single nuclei are diploid, hyperdiploid and tetraploid Most of the nuclei between 2c and 4c are not labelled Some nuclei show a haploid (1c) or hypertetraploid DNA content The DNA values of the whole cells range from the diploid (2c) to the octoploid (8c) level, according to the number of cells present The labelling index is 14%

Discussion

The main objectives of this study were (1) to investigate the proliferation pattern of LBC during the entire progress of the disease, and (2) to study the non-leukaemic haemopoietic cell systems in the preleukaemic stage

The results indicate that in the *LBC* the majority is diploid and a low proportion is hyperdiploid and tetraploid (fig 2) According to this 61–62% were found in G_1, 29–36% in S and 3–9% in G_2 A high percentage of diploid non-proliferating LBC in cases of acute leukaemia has

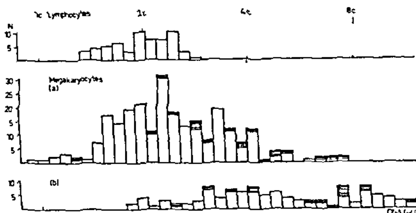


Fig 5 Relative DNA content (AU) and ^{3}H TdR labelling (shaded areas) of megakaryocytes in February 1970 (a) DNA *content of the single nuclei* (b) DNA content of the whole cells 1c 2c 4c 8c = mean haploid diploid tetraploid and octoploid DNA values N = number of cells or nuclei assessed

been observed by cytophotometry [13–15 23] and by combined application of cytophotometry and autoradiography [4 6] The range of ^{3}H TdR labelling indices *in vitro* given in the literature is extremely wide but in all reported series considerably lower (about 10%) than in normal dividing granulocytic cells (32–33%) [11] By use of the combined technique in normal human promyelocytes and myelocytes 61–62% were found in G_1 33% in S and 4–5% in G_2 [27] These data indicate that in the LBC of the present case the proportion of cells in the different cell cycle stages is similar to that found in normal dividing granulocytic cells and that high labelling indices are present compared to the majority of cases of acute leukaemia

At the different times of observation in the LBC no significant changes of the proliferation pattern were observed (fig 2) This is in contrast to the clinical course of a long preleukaemic phase of about 8 months and a short refractory phase of overt leukaemia lading to death within 5 months From the kinetic data obtained it seems to be unlikely that a change of proliferation activity of LBC is responsible for the rapid change in the progress of the disease In exponentially growing tumor cells a progressive decrease of the growth fraction has been observed with time [1 12] and in acute leukaemia the labelling index was found to be inversely related to the percentage of LBC in the marrow [5]

However, the results of the present case suggest no decrease of the proportion of DNA synthesizing cells during the progress of the disease Therefore, further studies seem to be necessary to understand the relationship between the kinetics of leukaemic cell proliferation and the clinical progress of the disease

In several studies evidence has been presented that the chance of remission and survival time increases with a high labelling idex [10, 22] From these observations a good response to treatment would have been expected in the present case However, the administration of several cytostatics as daunorubidomycine, vincristine, asparaginase and cytosine-arabinoside did not induce a remission This observation points to a further unusual manifestation of the disease in this case

The main characteristic finding during the preleukaemic phase is a striking proliferation defect of the erythropoietic and megakaryocytic cell system In *erythropoiesis* this proliferation defect was limited to the early polychromatic cells and consisted of an accumulation of cells in G_1 and a decreased proportion of cells in S (fig 3, 4) In normal human beings more than 60% of the polychromatic erythroblasts were observed in S by use of the same method [3, 19] The changes in the present case might be explained by a lack of induction of DNA synthesis in a large fraction of cells The reduction of the labelling index to 25% indicates a low effective production rate in the erythropoietic cell system, followed by a severe refractory anaemia

A similar proliferation defect in erythropoiesis has been recently described in cases of untreated acute leukaemia [7] and in other clinical manifestations of ineffective erythropoiesis as sideroblastic anaemia [25], congenital dyserythropoietic anaemia type II [18] and homozygous and heterozygous β-thalassaemia [16, 26] Therefore, the proliferation defect showing an increased G_1/S ratio in the early polychromatic erythroblasts refers to a common pathophysiological mechanism, resting on different etiological factors

In the *megakaryocytes* of the present case the morphologically apparent abnormalities (fig 1) suggest a severe dyspoiesis in this cell system In normal human megakaryocytes evidence has been provided for alternating times of DNA synthesis and resting periods from 4c–32c, indicating a rhythmical polyploidization [17] The changes found in the megakaryocytes studied indicate a restriction of the capacity for polyploidization leading only to octoploid (8c) cells (fig 5b) The observation of numerous unlabelled cells betwen 2c and 4c and between

4c and 8c suggests a marked disturbance of rhythmical polyploidization. In contrast to the normal state maturation occurs in many cells without polyploidization above the 4c level. Because the normal 16c–32c megakaryocytes are not present one has to assume that the pathological 4c–8c megakaryocytes are capable of an effective platelet production. In contrast to the erythropoietic cell line, the defect in the megakaryocyte production is not followed by peripheral thrombocytopenia (table I).

In chromosome studies of chronic myelocytic leukaemia [24] and in some cases of acute leukaemia [8] evidence has been given that the leukaemic process is not limited to one haemopoietic cell system. The results obtained in the present case suggest a defective erythropoiesis and megakaryocytopoiesis, which is already apparent at a time when LBC infiltration of the bone marrow is still minimal. Therefore, the preleukaemic stage may be defined as a period in which the basic proliferation defect of acute leukaemia becomes apparent largely in the so-called non-leukaemic cell populations, leading to peripheral mono- or pancytopenia.

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Acta haemat 47 33–42 (1972)

A Study of Haemostasis in Macroglobulinaemia

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Abstract In 3 patients with dysproteinaemia the defects in haemostasis were investigated Waldenstrom s macroglobulinaemia macroglobulinaemia associated with undifferentiated malignancy in the marrow diffuse hypergammaglobulinaemia associated with drug sensitive purpura Platelet factor 3 release reduction of its total contents and poor platelet adhesion were found in all 3 patients Platelet aggregation was absent in 2 patients who had macroglobulinaemia with monoclonal spike It is suggested that the platelet factor 3 assay may be of value in the investigation of these changes in relation to dysproteinaemias

Key Words
Coagulation
Dysproteinaemia
Haemostasis
Macroglobulinaemia
Platelet factor 3

A haemorrhagic diathesis is a frequent and sometimes serious complication in diseases associated with high concentrations of paraproteins [1–4] This group of disorders includes, among others, multiple myeloma, macroglobulinaemia and lupus erythematosus Haemorrhagic symptoms have been the presenting features occasionally in cases of multiple myeloma [5, 6]

Various mechanisms have been implicated in the bleeding tendencies of such patients In particular various types of qualitative platelet defects have been described [7–9] The presence of abnormal platelet factor 3 (PF_3) release has been shown to be due to coating of the platelets by abnormal globulins [10] PERKINS [8] however, did not find the decreased PF_3 release In 62 patients he described defective platelet adhesiveness and increased bleeding time

The present studies were done to evaluate platelet functions in 3 patients with dysproteinaemia We have shown defective platelet factor 3 release and adhesion in all the cases as well as abnormal agregation of

platelets in 2 cases In addition a method for quantitation of platelet factor 3 is described

Case Reports

The patients were attending the haematology clinic at the All India Institute of Medical Sciences between January 1966 and August 1970 They were diagnosed one each of Waldenstrom s macroglobulinaemia macroglobulinaemia with undifferentiated malignancy in bone marrow and drug sensitive purpura with diffuse hypergammaglobulinaemia

Case 1 This 50 year old female was first seen in April 1970 with chief complaints of exertional dyspnoea and cough for 3 years increasing weakness hoarseness of voice loss of hair and dryness of skin for 6 months and fever of 6 weeks duration On physical examination the pertinent findings were pallor and just palpable liver Laboratory examination revealed moderate anaemia Bone marrow aspiration showed decreased cellularity and collection of large number of cells of lymphoid series having PAS positive globules in the cytoplasm and nuclei Serum electrophoresis showed a monoclonal spike in gammaglobulin region which turned out to be IgM (table I) on immunochemical studies with κ light chains At this point a diagnosis of Waldenstrom s macroglobulinaemia was made 6 weeks later her platelet count was 180 000 mm³ without evidence of bleeding at any site Platelet function tests were done at this period and will be discussed separately Chlorambucil 4 mg and prednisone 40 mg p o daily along with supportive therapy was of no avail She died of massive generalised haemorrhage after 5 months of follow up Her terminal platelet count was 128 000 mm³ Autopsy showed haemorrhages at multiple sites particularly in the lungs

Table I Haemoglobin serum proteins and immunoglobulins

	Case 1	Case 2	Case 3
Haemoglobin g%	6 0	8 5	10 6
Total serum proteins g%	8 50	7 80	7 35
Albumin g%	2 26	3 33	3 79
Globulin g%			
α_1	0 31	0 06	0 04
α_2	1 07	0 60	0 11
β	1 03	0 17	1 06
γ	3 32	3 91	2 33
IgG mg/ml	16 25	8	14 00
IgA mg/ml[1]			1 50
IgM mg/ml	21 45	10	7

[1] IgA levels were not done in 2 patients

Case 2. This 5? year-old male patient was first seen in our clinic with chief complaints of pallor, weakness and petechial haemorrhages of 1 month duration. On examination he had severe pallor and moderate hepatomegaly. Blood examination showed pancytopenia. Bone marrow aspiration revealed a hypocellular ty without any abnormal cells. A serum electrophoresis showed a monoclonal spike in the γ-globulin region, which turned out to be mainly IgM (table 1) with κ light chains in immunochemical studies. A tentative diagnosis of aplastic anaemia with macroglobulinaemia was made at this point. On two different occasions during the follow up, the repeat bone marrow biopsies showed undifferentiated malignant cells in abundance. These cells did not contain PAS-positive globules. Initial treatment with androgens and prednisone resulted only in a temporary rise in haemoglobin noticeable within 10 days. However, the patient again became severely anaemic. Endoxan was also given for a brief period of 2 weeks prior to death. Macroglobulin levels did not come down during his course of illness. Platelet function studies were done after 4 months of follow up. At this time there was only moderate anaemia (haemoglobin 8.5 g%) with normal platelet (?90,000 mm³) and leucocyte counts (6,000 mm³). The total duration of illness was 9 months after his initial diagnosis in the hospital.

Case 3. This 56-year-old female patient presented in 1966 with chief complaints of echymotic patches off and on for one year, along with history of occasional bleeding at multiple sites, like gastrointestinal tract, joints and following tooth extraction. Blood examination showed thrombocytopenia (platelet count 56,000 mm³). Follow up was lost for one year. However, in 1967 she again complained of echymotic patches and related its association with aspirin, novalgin and equanil. However, her platelet count was normal at this time. She has now been followed for 3 years and did not show thrombocytopenia at any occasion. Platelet function studies were done in July 1970. Study of serum proteins was also done at the same time. A diffuse increase in γ-globulins was found in serum electrophoresis. Immunochemical studies revealed increased amounts of IgG and IgM (table I).

Methods

In each patient, total serum proteins and the paper electrophoresis were done [11]. The albumin and globulin concentrations were calculated therefrom. The platelet count was done using formal citrate red cell diluent [12]. Bleeding time was performed by MIELKE's modification of Ivy method [13]. LEE and WHITE [14] whole blood coagulation time, Quick one stage plasma prothrombin time [15], serum prothrombin time, prothrombin consumption index [16], clot retraction [17], partial thromboplastin time and activated partial thromboplastin time [18] were done in each patient.

Platelet adhesion was tested based on the method of BORCHGREVINK [19].

Platelet aggregation in platelet rich plasma was determined by a rapid qualitative test [20] after addition of ADP (10 μg/ml), adrenaline (1.25 μg/ml), noradrenaline (5.1 μg/ml) and thrombin (0.5 U/ml).

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β	1 03	
γ	3 32	
IgG mg/ml	16 25	
IgA mg/ml[1]		
IgM mg/ml	21 45	

[1] IgA levels were not done in 2 patients

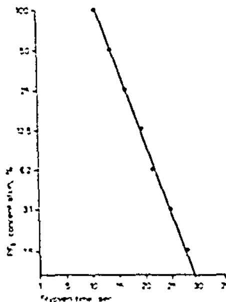


Fig 1 Stypven time (sec) of normal platelet poor plasma in relation to PF_3 concentration (%) of normal frozen and thawed platelet rich plasma

concentration and was absent The platelet adhesion was poor in all 3 patients

Platelet factor 3 release was abnormal as evidenced by a high stypven time at various time intervals (table IV) All patients had a prolonged stypven time of FTPRP as compared to the controls indicating the deficiency of PF_3 On assay PF_3 contents were found uniformly low in all 3 patients (table V) Further it was seen that patients PF_3 contents did not alter when suspended in normal plasma before being frozen and thawed Similarly normal platelets when suspended in patient's plasma remained normal This indicates that the estimation of PF_3 in the system was not altered by the presence of macroglobulins or increased globulins

Discussion

Numerous mechanisms have been used to explain the haemorrhagic phenomenon in dysproteinaemias Among those suggested have been

Table II Screening tests for haemostasis

	Case 1	Case 2	Case 3
Bleeding time, min	15	4	2 30
Coagulation time, min	12 30	11 15	9 30
Quick test, sec[1]	13 5/13 5	14/14	12/12
Clot retraction, %	30	58	42
Prothrombin consumption index %	74	46 6	52 2
Partial thromboplastin time, sec[1]	73/80	81/81	73/87
Activated partial thromboplastin time, sec[1]	38/41	46/45	41/47 5
Platelets/mm[3]	180 000	290 000	142,000

[1] Patient/normal

Table III Platelet adhesion and aggregation

	Case 1	Case 2	Case 3
Platelet adhesion %	2 3	19	0
Platelet aggregation			
adrenaline, sec	no aggregation	no aggregation	24 5/19 5
No adrenaline, sec	no aggregation	no aggreagtion	35/27 5
ADP, sec[1]	no aggregation	no aggregation	24 5/19 5
Thrombin sec	no aggregation	no aggregation	20 18 5

[1] Concentrations of ADP 0 6 μg and 60 μg/ml failed to produce platelet aggregation

thrombocytopenia [24], infiltration of small vessels of the vascular tree by abnormal proteins [25], capillary damage from impaired blood flow due to the often greatly increased blood viscosity [25], various types of qualitative platelet defects [7, 9], interference with fibrinogen to fibrin conversion [26–29], inhibition of various clotting factors [30, 31], fibrinolysis [32], coating of platelets by macroglobulins [10] and absorption of clotting factors by abnormal proteins [33]

The exact mechanism underlying defective haemostasis in these disorders is unclear Platelet abnormalities have been found to be of importance in our patients The defective PF_3 release and its deficiency is striking WEISS [34] used thromboplastin generation test to study the PF_3 release and showed its abnormality in patients with paraproteinae-

Table IV. PF3 release at 37°C after incubation with ADP

	Incubation time, min				
	0	5	10	15	20
Clotting time, sec	27.0	21.5	22.5	18.0	14.0
Normal	44.5	41.0	41.0	17.4	34.0
Case 1	21.0	21.0	23.5	17.5	14.0
Normal	31.5	30.0	21.0	27.0	25.5
Case 2	32.0	28.0	21.0	25.5	16.0
Normal	31.5	34.0	33.0	31.5	31.5
Case 3	—				—

Table V. Sedimentation time of ETPRP and PF3 assay

	Case 1	Case 2	Case 3
Sedimentation time of ETPRP, sec (patients/control)	11.5/7.5	12.5	11.5/11.0
PF3 assay, percent of control	57.0	30.0	44.6

mas. However, till recently this has not been confirmed [8, 9]. Our studies suggest that the platelet factor 3 was not only poorly released but total contents were reduced (table V). The diminished release of PF_3 in these patients could be due to their reduced contents of PF_3 [35]. However, the proportionate release of PF_3 was much less in patients than in controls. At 20-minute intervals the PF_3 released from platelets were 1% (case 1), 2.5% (case 2) and 12% (case 3) in patients as compared to 35, 38 and 19%, in respective controls, thus confirming that there is defective release as well as reduced contents of PF_3. This could be due to the coating of platelets by abnormal serum proteins as suggested by PACHTER [10]. However, the inability of plasma from such patients to reduce the PF_3 activity of normal platelets would suggest that the abnormal proteins did not interact with PF_3 released by freezing and thawing. An intrinsic platelet defect is another possibility. The reduced total contents of PF_3 in the platelets of these patients would support this postulation. The total amount of PF_3 in patient's platelets remained low when suspended in normal plasma before disruption. This would again support the fact that there was true PF_3 deficiency.

The presence of immunoglobulins inside the platelets may interfere with the PF_3 activity and account for its poor release and reduced total contents, however, it remains to be shown Immunoglobulin G has been shown to be associated with the platelets [36] but not the IgM

ADP was employed to induce the PF_3 release from the platelets and in the absence of aggregation in two cases the failure of membrane changes preceding the release of PF_3 can be incriminated as a possible mechanism [21] However, diminished PF_3 release with normal aggregation (case 3) would not support this possibility

We have shown reduced *in vivo* platelet adhesion in these patients which is consistent with other observations [7, 8, 9]

Bleeding time has been found to be abnormal in only one patient (case 1), although platelet aggregation was absent in both the patients with macroglobulinaemia Normal bleeding time with absence of platelet aggregation in cases of macroglobulinaemia has already been shown by ROZENBERG and DINTENFASS [37]

Absence of platelet aggregation along with defective release of PF_3 and reduced total content in these patients would suggest an acquired compound platelet abnormality, which can be designated as acquired thromboasthenic deficit thrombopathy

Acknowledgement The authors wish to acknowledge the help of Dr A MANGALIK Assistant Professor of Medicin All India Inst tute of Medical Sciences for permitting us to study his patients We also wish to acknowledge Dr A N MALAVIYA Assistant Professor of Med cine AIIMS for the analysis of serum immunoglobulins in these pat ents The authors are also grateful to Dr WILLIAM A ROBINSON Assistant Professor Colorado University Med cal Center Denver USA for his critical comments on th s study

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Acta haemat 47 43–48 (1972)

Hemoglobin J Paris (α12 Alanine→Aspartic Acid) in Two Iranian Families

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Key Words
Hemoglobin J Paris
Hemoglobinopathies

Abstract Hemoglobin J Paris (α12 Ala→Asp) was found in 2 unrelated families in Iran during a survey among hospitalized patients No erythrocytosis or leukocytosis were present in the heterozygous carriers contrary to what was observed in the first case in France A modified technique is given for the preparation of the peptide chains of Hb J

Hemoglobin J (α12 Ala→Asp) was first described by ROSA *et al* [13] in a woman of Spanish origin in Paris and has since been found in Portugal in a population with possibly Arab connection [15] Since then it has also been found in a British citizen of Indian origin [Prof H LEHMANN, personal communication]

We have recently found this hemoglobin in members of 2 Iranian families in the course of a survey for abnormal hemoglobins, which resulted in finding of a number of known and unknown hemoglobins [10, 11] No clinical symptoms were associated with this abnormal hemoglobin in the carriers which all were heterozygous for Hb A–Hb J, unlike the erythrocytosis and leucocytosis found by ROSA *et al* [13] in their patient

Methods and Results

Routine hematological examinations performed as standard technics [17] were within normal range, RBC 4,500,000–5,000 000, WBC 8,000–9,000/mm³ Hemoglobin between 15–16 g/100 ml No inclusion bodies found in the red cells Heat denaturation was normal

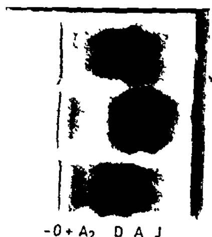


Fig 1 Starch gel electrophoresis pH 8 6 Upper Hb A–Hb Q (Q Iran) Middle Hb A–Hb J Lower Hb A–Hb D

Hemoglobin electrophoresis performed on cellulose acetate [5] and in starch gel with tris-EDTA-borate buffer pH 8 6 showed a fast-moving hemoglobin fraction in addition to the normal, Hb A (fig 1) The fast-moving fraction had a mobility similar to Hb J Iran [10] The proportion of Hb J α was estimated by elution of Hb J band from cellulose acetate after electrophoresis [9] to be 26% of total hemoglobins This hemoglobin moves with Hb A in the agar gel electrophoresis [12]

In starch gel electrophoresis there was a second fast Hb A_2 suggesting that the abnormality exists in the α-chain This was confirmed by the treatment of the hemolysates with *p* mercuribenzoate (PMB) [1] followed by starch gel electrophoresis pH 8 6 which revealed an additional fast moving α-chain (fig 2)

Separation of the globin chains by starch gel electrophoresis containing 6 M urea and 0 05 M 2-mercaptoethanol [2], as well as cellulose-acetate electrophoresis with 6 M urea buffer [16] confirmed that the abnormality was confined to the α-chain of Hb J Hemoglobin J was purified by column chromatography on DEAE Sephadex [8], using tris-HCl buffers ph 8–7 2, and concentrated *in vacuo* Globin was prepared with 2% concentrated HCl in acetone at –20 °C, washed with cold acetone and freeze-dried

Peptide chains of Hb J were prepared by CMC column chromatography of the globin with 8 M urea and 0 05 M 2-mercaptoethanol in phos-

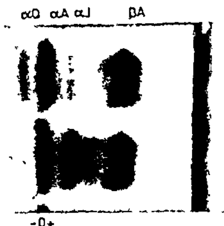


Fig 2 Starch gel electrophoresis pH 8.6 from PMB treated hemoglobins Upper PMB chains from Hb A Hb Q with a slow moving α-chain Lower PMB chains from Hb A Hb J with a fast moving α-chain

phate buffer of pH 6.7 [3] except that the aminoethylation of the peptide chain was omitted

Separation of the peptide chains of Hb J was accomplished also by the treatment of the hemolysates with PMB [1], followed by DEAE cellulose chromatography (Whatman DE-52) In this method fresh hemolysates without separation of Hb J from Hb A were treated with PMB [4], and after overnight incubation in cold (4 C) PMB peptide chains were dialysed against tris-HCl buffer pH 8.1 0.05 M for 4 h and applied to the DE-52 column equilibrated with the same tris-HCl buffer used for dialysis The column was eluted with the same equilibration buffer, the α-PMB of Hb J elutes subsequent to the α-PMB chain of normal Hb A

Tryptic digest of the α-chain globin and fingerprinting of the normal and abnormal α-chains was done according to CLEGG *et al* [3] In the fingerprint from the soluble peptides of Hb J, the αTp3 peptide is missing from its normal position (fig 3, 1) and an additional peptide was found below the normal αTp4, near the origin which stained *for tryptophane* [14]

Preparative fingerprints were prepared, each with 3 mg of soluble tryptic peptides and the aberrant peptide was eluted with 0.5 N NH_4OH

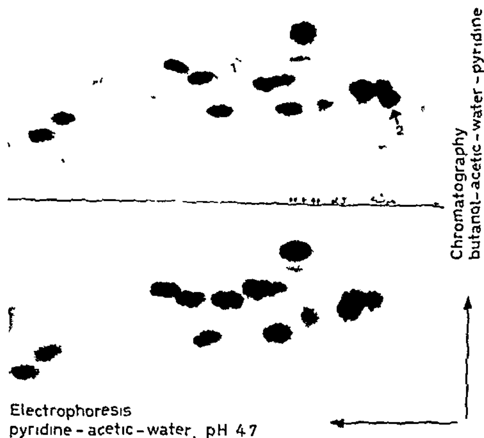


Fig 3 Fingerprints from soluble tryptic peptides of a normal α chain (lower) and an α chain from Hb J (upper) (1) The normal αTp3 is missing (2) An additional Tryptophane positive peptide is seen

and freeze-dried Part of the αTp3 peptide was hydrolyzed with 6 N HCl at 105 °C for 18 h and its amino acids were determined in an automatic amino acid analyser (EEL, model 106)

Amino acid composition of the αTp3 peptide of Hb J was 1 lysine, 1 alanine, 1 glycine, and 1 aspartic acid, suggesting the substitution of an aspartic acid for an alanine (tryptophane was destroyed completely during acid hydrolysis) Since the amino acid sequence of the normal αTp3 is

12 13 14 15 16
Ala – Ala – Try – Gly – Lys

to decide which of 2 alanine residues, 12 or 13, was substituted by the aspartic acid N terminal determination by the Dansyl method of GREY and HARTLEY [6], was performed on the αTp3 of Hb J and the DNS amino acid was distinguished by 2-dimensional thin layer chromatography on Polyamide sheets using reference DNS amino acids as control, in this way the N terminal amino acid found to be aspartic acid in the αTp3 from Hb J, the original N terminal amino acid was removed from the peptide by the 'combined Edman Dansyl method of GRAY [7] and the subsequent residue found to be alanine, the sequence of the abnormal peptide should be Asp – Ala – Try – Gly – Lys

Acknowledgment We are grateful to the MRC of Great Britain for providing us with the amino acid analyser used in this work.

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17 WINTROBE, M M Clinical hematology, 5th ed (Lea & Febiger, Philadelphia)

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Acta haemat 47 49–58 (1972)

Effect of Tilorone HCl, an Oral Interferon-Inducer, on Leukopoiesis in Rats

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Key Words
Interferon
Leukopoiesis
Lymphatic reaction
Tilorone

Abstract Single oral doses of 100 mg/kg T to rats caused marked lymphopenia Recovery started after 12 h and was accompanied by depletion of small lymphocytes in lymph follicles of the spleen and the appearance of young lymphocytes with vacuoles and basophil granules in smears of lymph nodes and spleen Abnormal mononuclear cells whose protoplasm often contained vacuoles and basophil granules appeared in the blood after 48 h They disappeared in the course of 2–3 weeks The granulocytic system responded to T with a short lasting leukocytosis a shift to left and appearance of basophil granules in neutrophil and eosinophil promyelocytes and myelocytes Repeated administration of T caused only minor hematologic changes. No other organs were affected by the drug

Tilorone HCl (2,7-bis[2 (diethylamino)ethoxy]fluoren 9-one) is an orally active inducer of interferon [6] It protected mice against a variety of infections with DNA and RNA viruses [3] During the preliminary toxicologic evaluation ROHOVSKY *et al* [7] discovered the appearance of abnormal mononuclear cells in the peripheral blood of laboratory animals Basophil granules and vacuoles were often present in the protoplasm of these cells Similar changes were seen in cells of the reticuloendothelial system In this paper the effects of single and multiple doses of tilorone HCl (T) on leukopoiesis of rats was analyzed through the combined use of hematologic and histopathologic techniques

Methods

CFN/Zu rats of both sexes weighing from 122 to 180 g were used They were housed individually in Macrolone cages A commercial diet (Nafag Gossau) and

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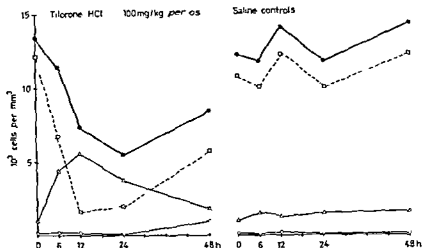


Fig. 1. Hematologic effects of a single oral dose of 100 mg/kg T and of physiologic saline. ● Total white cell count, □ lymphocytes, △ neutrophile leukocytes, ○ monocytes and abnormal mononuclear cells. Each point represents the mean of 3 rats which were sacrificed after blood was taken. Initial counts are means of all animals used in the experiment.

Results

Single Oral Administration

100 mg/kg T caused a rapid decrease in lymphocyte count. The lowest level, less than 2,000 cells/mm³, was reached after 12 h. Simultaneously, the neutrophil granulocytes rose markedly and showed a prominent shift to left. 48 h after T these changes had partially reverted to normal (fig. 1). At this time blood contained 6–15% abnormal mononuclear cells. These were quite large (14–25 μm) and had a round, somewhat excentric, often kidney-shaped or lobulated nucleus. The protoplasm was light blue, it often contained basophil granules. Small vacuoles were seen in about one quarter of these cells (fig. 2a–c). The granules had an affinity for Sudan black. In cells without granules there was no Sudan black positive material (fig. 3a, b). In the bone marrow the percentage of mature neutrophil granulocytes was reduced at 12–48 h. The myeloid-erythroid ratio was shifted in favor of the red cell line at 24 h. At this time the neutrophil and the eosinophil promyelocytes and myelocytes contained coarse basophil granules (fig. 4). These were still present in some cells after 48 h. In smears of the spleen there was an increase in young lymphocytes, particularly 6 h, and to a lesser degree

Table I

Treatment	Number of Rats		Dose, mg/kg	Route	Hematologic examinations
	treated	control			
Single administration	12	12	100	*per os*	before, 6, 12, 24, 48 h after T
Single adminstration	8	8	100	*per os*	before, 24, 48 h after T
Single adminstration	18	18	100	i p	before, 2, 3, 6, 9, 13, 16, 23 days after T
Single administration	3	3	100	*per os*	before, 3, 6, 9, 12, 24 h after T eosinophil counts
Single administration (adrenalectomized)	5	5	100	*per os*	before, 3, 6, 9, 12, 24 h after T
Repeated administration	6	6	2×100	*per os*	
Repeated administration	10	8	8×30	*per os*	before, after 5 and 8 doses

water were supplied *ad libitum* A group of 10 rats was adrenalectomized and maintained on 1% NaCl in the drinking water Blood was obtained from the tail vein Leukocyte and eosinophil counts were done by conventional technique Blood smears were stained with May-Grunwald Giemsa and Sudan black [2] At autopsy a bone marrow cylinder was taken from the femur, suspended in rat serum and spread on polished glass slides Smears were also made from the spleens and axillary lymph nodes The histopathologic examination included spleen, bone marrow (sternum), thymus, inguinal, axillary and mesenteric lymph nodes, liver, lung, pancreas, small and large intestine, adrenal gland urinary bladder and testis Tissues were fixed in buffered formaline, embedded in paraffine, sectioned and stained with hematoxylin eosin Spleen and thymus weights were determined in one group 24 h after 100 mg/kg T *per os* Rectal temperature was measured before, 3, 6, 9, 12 and 24 h after 100 mg/kg T *per os* with an electrical Ellab TE 3 Universal thermometer

T was dissolved in distilled water and administered by stomach tube or in saline for intraperitoneal (i p) injection Control animals received equal volumes of physiologic saline The treatment schedules are shown in table I

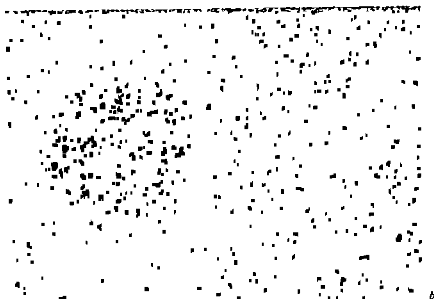


Fig 5 Spleen, rat H E, ×100 *a* Lymph follicle of a saline treated control *b* Lymph follicle 24 h after 100 mg/kg T *per os* Marked depletion of lymphocytes in the area surrounding the central artery

cytes in the areas surrounding the central arteries (fig 5a, b) These changes were not yet present in animals sacrificed after 6 h They were moderate after 12 h and marked after 24 h 48 h after T the spleen appeared unchanged No abnormal changes were seen in all other organs examined

Eosinophil Counts and Rectal Temperature after Single Oral Administration

100 mg/kg T did not cause a change of the eosinophil counts (table II) Rectal temperature was slightly decreased 6 and 9 h after treatment (table II)

Single Oral Administration in Adrenalectomized Rats

The effect of T on the white blood cells was similar to that described for nonadrenalectomized rats The decrease in lymphocytes however, was delayed Minimal lymphocyte levels were reached 24 h after T administration

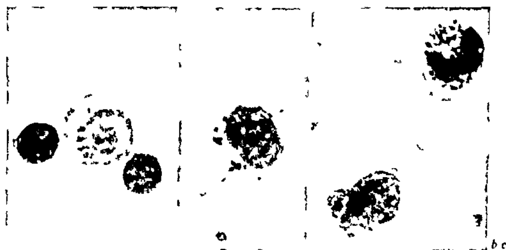


Fig 2 Blood smear of a rat 48 h after 100 mg/kg T *per os* May Grunwald Giemsa × 1 000 *a* Large mononuclear cell without granules Some rarefication of the protoplasm near the nucleus Two mature lymphocytes *b* Mononuclear cell with basophil granules *c* Two mononuclear cells with kidney shaped nucleus One cell with granules and vacuoles

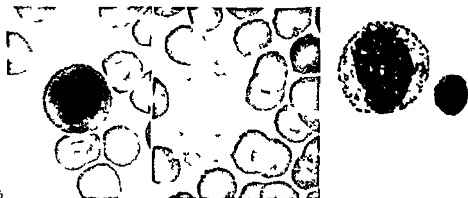


Fig 3 Blood smears of rats Sudan black × 1 000 *a* Monocyte of untreated rat high affinity of protoplasm for Sudan black *b* Mononuclear cell 48 h after 100 mg/k gT *per os* Protoplasm with vacuoles Sudan black negative

Fig 4 Bone marrow smear of rat 24 h after 100 mg/kg T *per os* May Grunwald Giemsa Eosinophil metamyelocyte (ring shaped nucleus) with coarse basophil granulation

also 12 and 24 h after T Some of these contained basophil granules. The weight of the spleen and thymus 24 h after treatment was not different from that of the controls The histopathologic evaluation of the spleen revealed hyperemia of the red pulpa and depletion of lympho-

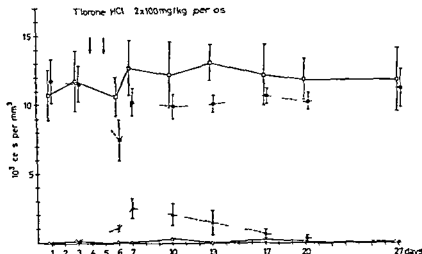


Fig 7 Hematologic effects of 2×100 mg/kg T *per os* in rats ■ Lymphocytes of T treated rats □ Lymphocytes of saline treated rats ▲ Monocytes and abnormal mononuclear cells of T treated rats △ Monocytes of saline treated rats Mean of 6 rats ± SD

however a discrete shift in cell composition was noted There was a slight increase in young lymphocytes and abnormal mononuclear cells, particularly after 24 and 48 h Many of these cells contained small basophil granules often also small vacuoles (fig 6) Such cells were rarely seen in smears of untreated rats The histopathologic examination of the spleens showed hyperemia of the red pulpa at 12 h At 24 h hyperemia was more prominent There was severe depletion of the lymphocytes in the tissue surrounding the central artery and a narrowing of the marginal zone of the lymph follicles At 48 h small lymphocytes were again present at the central zone 1–2 days later the structure of the spleen was normal

Survival of the Abnormal Mononuclear Cells in the Blood (fig 7)

After 2 oral doses of 100 mg/kg T the number of abnormal mononuclear cells reached appreciable levels with a maximum 48 h after the second dose These cells were cleared from the blood in 2–3 weeks The rats were sacrificed 33 days after treatment The histopathologic examination of various organs revealed no abnormal findings

Table II Eosinophil counts and rectal temperature of rats treated with 100 mg/kg T *per os* or saline

Time	Eosinophil counts[1]		Rectal temperature[1]	
	treated	controls	treated	controls
Before treatment	129 (40–227)	96 (67 127)	38 0 (37 5 38 7)	38 2 (38 0–38 4)
3 h	116 (40–187)	27 (13 40)	37 6 (37 4 37 8)	37 8 (37 6–37 9)
6 h	145 (67 200)	42 (27– 67)	36 6 (35 5 37 0)	37 4 (37 2 37 6)
9 h	156 (67 267)	76 (53 107)	36 7 (35 5 37 2)	37 5 (37 4 37 7)
12 h	156 (67 227)	111 (67–173)	37 2 (36 8 37 9)	37 4 (37 0–37 8)
24 h	277 (153–400)[2]	112 (80 167)[2]	37 7 (37 7–37 8)	37 3 (37 1 37 6)

[1] Means of 3 rats and range
[2] One animal died accidentally

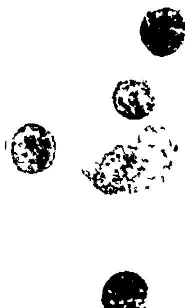


Fig 6 Smear of axillary lymph node of a rat 24 h after 100 mg/kg T *per os* Mononuclear cell with small basophile granules and 4 mature lymphocytes May Grunwald Giemsa ×1 000

Effect of Single i p Injections on Lymph Nodes and Spleen

Rats were sacrificed 6–96 h after one i p injection of 100 mg/kg T The histopathologic examination of subcutaneous and mesenteric lymph nodes revealed no consistent changes In differential counts on smears

untreated rats contained abundant Sudan black positive material Considering all evidence it is felt that the abnormal mononuclear cells appearing in the blood 48 h after T administration belonged to the lymphatic cell line

The appearance of large mononuclear cells (transformed' lymphocytes, lymphoblasts, reticulum cells) was also observed in mice treated with macromolecular interferon inducers such as bacterial double-stranded RNA and poly IC (= a complex of polyinosinic acid and polycytidylic acid) [4, 5] It is possible, therefore, that interferon induction and stimulation of the lymphatic system may, in some way, be linked The double-stranded RNA of LEONARD *et al* [4], however, caused not only a depletion of lymphocytes in the spleen but also in the thymus T had no effect on the thymus which might be explained by its lack of systemic toxicity

The neutrophil granulocytes responded to a large dose of T with a sharp increase with a maxium at 12 h and a marked shift to left The nonsegmented and segmented polymorphonuclear cells of the bone marrow were decreased The immature precursors of the neutrophils also contained coarse basophil granules, but the mature granulocytes of the peripheral blood did not show basophil material Coarse basophil granules were particularly abundant in promyelocytes and myelocytes of the eosinophil cell line In the circulating blood eosinophils were numerically and morphologically normal

The significance of the basophil granules in lymphatic cells and granulocyte precursors is not known They might be considered as a consequence of incomplete or disturbed maturation The immature and abnormal cells of the lymphatic cell line were washed out into the circulating blood in response to the marked drug induced lymphopenia It is probable that they were structurally deficient and, therefore, removed from the blood within 2–3 weeks The neutrophil and eosinophil leukocytes completed their maturation in the bone marrow

The acute effect of T seemed to be specific for the hematopoietic system No changes were found in other organs in particular the rapidly dividing epithelium of the testis and intestine There was no effect on the fixed reticuloendothelial system In the experiment of ROHOVSKY *et al* [7], these cells were found to be markedly affected by T The reason for the discrepancy cannot be determined

The single large dose of T which caused profound changes of the lymphopoietic and leukopoietic systems did not adversely affect growth and

Repeated Oral Administration

Rats tolerated 8 doses of 30 mg/kg well. Weight gain was not significantly influenced. After 5 doses the blood contained 11.2 ± 9% of abnormal mononuclear cells. They decreased to 3 ± 2% after 8 doses. There were no significant changes in total white cell and eosinophil leukocyte counts. After 5 doses only 3 of 10 treated rats showed a slight increase in neutrophil granulocytes with a concomitant decrease in lymphocyte counts. All animals had normal differential counts after 8 doses. Differential counts on smears of spleen and bone marrow revealed no differences between treated and control animals. The histopathologic evaluation of the various organs did not disclose any abnormal changes. In particular, there was no accumulation of basophil material in cells of the fixed reticuloendothelial system.

Discussion

The most noteworthy hematologic effect of a single large dose of T was a rapid loss of more than 80% of the circulating lymphocytes. Minimal counts were reached at 12 h, after which lymphocytes were replenished swiftly. The histologic evaluation of the spleen revealed a depletion of small lymphocytes in the central areas of the lymph follicles. No consistent changes were seen in lymph nodes and thymus. It is probable, therefore, that most of the lymphocytes reappearing in the blood between 12 and 48 h were mobilized from the lymph follicles of the spleen. The first evidence of response of the splenic lymph follicles was seen at 12 h, it was most prominent at 24 h and essentially over at 48 h. It is interesting to note that the antiviral effect of T after oral administration in mice followed exactly the same time course [6], thus, maximal antiviral activity coincided with the most active phase of replenishment of circulating lymphocytes.

T not only affected the mature lymphocytes but also their precursors. Immature lymphocytes with basophil granules and vacuoles were found in smears of lymph nodes and spleen 24 h after T administration. After another 24 h abnormal mononuclear cells whose protoplasm often contained vacuoles and basophil granules appeared in the circulating blood. The basophil granules had an affinity for Sudan black. The protoplasm of the abnormal mononuclear cells not containing granules was Sudan black negative. In contrast, the rare monocytes occurring in the blood of

Acta haemat 47 59–64 (1972)

The Iso-Electric Fractionation of Rabbit Ferritin

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Abstract Using the iso-electric focusing technique microheterogeneity of normal rabbit ferritin has been demonstrated The iso-electric points of the sub-fractions are 5 18, 5 15 5 00 and 4 95 Evidence for the existence of a variant of normal rabbit ferritin is presented and the value for the iso-electric point of this abnormal ferritin is 4 46

Key Words
Ferritin fractions
Iso-electric fractionation
Rabbit ferritin

Ferritin is an iron containing protein which functions both as a depot for the storage of iron and as a regulator of the iron absorption from the gut [1] It has been isolated from several organs, as for instance from the liver, spleen, bone marrow and reticulocytes [2]

From the work of Alfrey *et al* [3] it has become clear that organ-specific ferritin exists Ferritins isolated from human liver and spleen migrate with different mobilities when subjected to electrophoresis on celluloseacetate strips Recently Drysdale *et al* [4] showed that a horse spleen ferritin preparation could be fractionated into a number of distinct bands using a technique of iso-electric focusing on acrylamide gels Since we are interested in the properties of rabbit liver ferritin [8, 9], we tried to fractionate rabbit liver ferritin by means of the iso-electric focusing technique on a sucrose gradient as carrier In 5 out of 6 rabbits we found only one ferritin fraction and in 1 rabbit two ferritin fractions with the widely different iso electric points

Materials and Methods

All chemicals used were of analytical reagent grade

The rabbit liver ferritin was prepared in the following way The liver of a rabbit obtained from the slaughter house was homogenised in a borate buffer pH 8 6

development of the experimental animals In order to exclude a stress effect [1] blood eosinophils were counted There was no drop in eosinophil counts after a single oral dose of T. Rectal temperature was not raised In adrenalectomized rats T also caused a decrease in lymphocytes and an increase in neutrophil leukocytes with marked shift to left The only difference between adrenalectomized and nonoperated animals was a delay of about 9 h before the lymphocytopenia developed

Acknowledgements We thank Dr J W NEWBERNE of the Wm S Merrel Company, Cincinnati Ohio, for a generous supply of Tilorone HCl, Miss E BAUMGARTNER, Mr C HAEBERLIN and Mr H NEF for technical assistance, and Dr W H WEIHE for supply of the adrenalectomized rats

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Acta haemat 47 65–70 (1972)

Fibrinolysis in Myelomatosis

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Abstract The fibrinolytic system has been studied in 12 patients with myelomatosis Ten had some impairment of their fibrinolytic mechanisms and 4 of these had laboratory evidence of intravascular coagulation Defective fibrinolysis may be an important factor in the pathogenesis of thromboembolic phenomena in myelomatosis

Key Words
Fibrinolysis
Intravascular coagulation
Myelomatosis
Plasmocytoma

Thromboembolism is not considered a frequent complication of myelomatosis CATOVSKY *et al* [5] reported an incidence of about 3% of 376 patients, whilst fatal pulmonary embolus accounted for about 3% of all deaths in their series We believe that this underestimates the true incidence of thromboembolic phenomena in patients with myelomatosis We ourselves have recently observed thrombotic episodes in 3 of 12 patients with this condition

Although many factors may be implicated in the pathogenesis of thrombosis there is evidence which suggests a possible causal relationship between vascular occlusion and a defective fibrinolytic system [7, 8] In view of this, and because of our own observations of thromboembolic phenomena in patients with myelomatosis, we have examined the fibrinolytic system in 12 such patients and have attempted to relate the findings to their clinical status

Patients

Twelve patients, 10 male and 2 female, aged 38–70 years, were studied In each case, the diagnosis of myelomatosis was made on the presence of at least 2

Table I Details of subjects studied

Case No	Sex	Age years	Para protein	Time since diagnosis months	Treatment	Thromboembolic phenomena
1	M	38	IgGL	38	cyclophosphamide	deep vein thrombosis
2	M	55	IgGK	22	melphelan	
3	M	54	IgAK	26	cyclophosphamide	
4	M	64	IgGK	41	cyclophosphamide	
5	*M*	*55*	*IgGK*	*0*	*none*	*renal failure fibrin in* glomerular capillaries
6	F	65	IgAL	28	none	
7	F	58	IgGK	18	none	
8	M	66	IgGL	45	melphelan	
9	M	68	IgAK	56	cyclophosphamide	pulmonary embolus
10	M	70	IgGL	19	intermittent prednisolone melphelan	deep vein thrombosis
11	M	48	IgGL	12	intermittent prednisolone melphelan	
12	M	66	IgGK	42	none	

of the following 3 criteria (a) diagnostic bone marrow, (b) biochemical demonstration of a monoclonal gammopathy (c) osteolytic skeletal lesions

Techniques

Plasma fibrinogen was measured by the method of ELLIS and STRANSKY [6] Normal levels are 200–400 mg/100 ml

Euglobulin lysis time was estimated by the method of BUCKNELL [4] The euglobulin lysis time is expressed in units by multiplying the reciprocal of the lysis time in minutes by 10 000 The normal range is 66–110 units Plasma plasminogen was estimated by the method of REMMERT and COHEN [17] as modified by ALKJAERSIO *et al* [1] The normal range is 2 0–4 0 units/ml

Serum fibrin degradation products (FDP) were estimated by using the Burroughs Wellcome kit The sensitivity of the method is 1 25 μg/ml The normal range is 1 25–10 0 μg/ml

Fibrinolytic inhibitors of urokinase were estimated by the method of MCNICOL *et al* [12] The normal is 8–21 min

Factor VIII was assayed by a modification of the method of BIGGS *et al* [3] Normal values >50%

Factor V was assayed by the method of QUICK and STEFANINI [16] Normal values >50%

Table II. Summary of results in 12 patients [illegible]

Case No.	Euglobulin lysis time (min)	Plasminogen (units/ml)	[illegible]	Fibrinogen (mg/100 ml)	FDP (µg/ml)	Factor VIII %	Factor V %
1	33	2.8	25	240	10	102	93
2	57	2.7	14	-	10	120	95
3	115	2.7	30	178	10	62	-
4	27	4.8	31	600	40	65	121
5	80		120		320		
6	45	4.0	39	425	10		
7	33	4.5	25		10	100	105
8	65	3.2	13	470	-	75	-
9	29	1.9	14		27.5	170	60
10	41	2.6	45		10	93	93
11	33	2.8	45	600	22.6	95	40
12	47	1.5	93	310	10		
Normal controls	64–166 (25)	2.0–4.0 (25)	10–21 (25)	250–450 (25)	0–10 (30)	50	50

Results

Plasma fibrinogen was measured in 7 patients. Only 2 patients had a normal level. In 2 subjects the plasma fibrinogen was markedly elevated. Both of these had significantly reduced activator levels. Two patients had a marginally-elevated plasma fibrinogen whilst in the remaining individual this parameter was slightly reduced.

Plasma plasminogen activator. Euglobulin lysis time was prolonged in 10 of the 12 subjects. It is perhaps significant that those patients with the longest euglobulin lysis times had increased fibrin breakdown products in their serum. Moreover, 2 of these patients had plasma fibrinogen levels of 600 mg/100 ml.

Plasma plasminogen was normal in 9 of the 11 subjects in whom it was measured. The remaining 2 subjects had increased plasminogen levels.

Serum fibrin degradation products were elevated in 4 patients. All 4 had reduced activator activity and 3 increased activator inhibitor activity.

Fibrinolytic inhibitors of urokinase Activator-inhibitors were increased in 9 of the 12 patients in whom they were measured

Clotting factors V and VIII were normal in all patients in whom they were measured

Discussion

Throughout life there is continual deposition of fibrin on vascular endothelium which has been damaged by the stresses of the circulatory flow [9] Following repair and serving to maintain the patency of the vascular tree, the fibrin is removed by the proteolytic action of the fibrinolytic enzyme system This is a complex system whose structure closely resembles that of the coagulation system with which it is in dynamic equilibrium Essentially, plasminogen, an inactive globulin, is converted to plasmin, a powerful proteolytic enzyme which is able to digest fibrin with the release of fibrin degradation products

It is apparent that a defective fibrinolytic system may result in some impairment in the removal of fibrin from the vascular endothelium The patency of the vascular tree is thus threatened and thrombosis becomes a possibility This concept receives some clinical support from the observations of ELLISON and BROWN [7], who reported reduced fibrinolytic activator activity in a group of patients who had had a pulmonary infarction within the preceeding year

Reduced fibrinolytic potential may result from reduced activator activity, as measured by euglobulin clot lysis, or from an increase in activator-inhibitor activity, as measured by the urokinase sensitivity test In this study both tests were abnormal in a significant number of the patients examined In fact, only 2 of the 12 had a normal euglobulin clot lysis time and only 3 of the 12 a normal urokinase sensitivity test

It is possible that the prolongation of the euglobulin clot lysis time in myelomatosis is a reflection of the abnormal fibrin structure rather than a reduction of activator activity Abnormal fibrin polymerization and morphological abnormalities of the fibrin network have been reported by a number of workers [10, 11, 15], and it may be that structually abnormal fibrin is more resistant to the proteolytic activity of plasmin, particularly as it has also been noted that irregular aggregates of amorphous material may cover the abnormal fibrin strands [11] The increased resistance will be reflected by prolongation of the euglobulin clot lysis time It must be pointed out, however that the ultrastructural

studies of LACKNER *et al* [11] in 2 patients with myelomatosis revealed a marked reduction in the width of the abnormal fibrin strands and it is equally feasible that these would be more susceptible to lysis than normal ones.

Increased plasma fibrinogen concentrations have been previously recorded in patients with myelomatosis and other malignant conditions [13, 14]. In the context of thromboembolic phenomena this is of some significance since it has been shown that, provided there are no compensatory changes of the fibrinolytic system increases in fibrinogen concentration are associated with an increased resistance of fibrin clots to lysis [2].

It is of interest therefore, that 2 patients in this study each had a plasma fibrinogen concentration of 800 mg/100 ml. In addition, activator activity was markedly reduced whilst fibrin(ogen) degradation products were increased in both patients. Since active primary fibrinolysis is excluded by normal plasminogen levels and prolonged euglobulin clot lysis times it would appear likely that the elevated fibrin degradation products are derived from local fibrinolysis of fibrin deposits and it may be that these findings reflect low grade intravascular coagulation. Fibrin degradation products were also elevated in 2 other individuals one of whom presented with acute renal failure with demonstrable fibrin in the glomerular and intertubular capillaries and the other died 4 months later from a clinically-diagnosed pulmonary embolus.

Thus 10 of the 12 subjects with myelomatosis had some impairment of their circulating fibrinolytic activity and 4 of these had elevated fibrin degradation products which probably reflects local fibrinolysis secondary to the deposition of fibrin. In one of these patients marked intraglomerular fibrin deposition was demonstrated by a renal biopsy. It is considered that these results predispose to thomboembolic phenomena and we agree with CATOVSKY *et al* [5] that the incidence of thromboembolism in myelomatosis has been underestimated.

Acknowledgements. Our thanks are due to Mr. R. MALLS for valuable technical assistance and to Prof. E. K. BLACKBURN for helpful criticism and advice.

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Acta haemat. 47 71–80 (1972)

L-Asparaginase Effect on the Erythrocyte-Immunoglobulin Binding in Acquired Hemolytic Anemia

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Abstract. The possibility of direct release of immunoglobulin from the erythrocyte surfaces due to the break of the erythrocyte immunoglobulin binding after the i.v. administration of L asparaginase has been shown in patients with acquired hemolytic anemia of IgG and IgA type. These studies were carried out by means of immunofluorescent observation, the determination of the immunoglobulin content in the serum and the direct antiglobulin test

Key Words
Asparaginase
Erythrocyte antibodies
Hemolytic anemia
Immunoglobulins
Immunofluorescence

It was shown that L asparaginase (Asn ase) administration to patients with acquired hemolytic anemia may improve the course of the disease when administered at high doses [19], whereas small doses enhance the erythrocyte destruction in peripheral blood [31]. This paper illustrates our study on the Asn ase effect on the behavior of immunoglobulins coating erythrocytes, and on the behavior of the serum immunoglobulins, in patients with acquired hemolytic anemia of IgG and IgA type. The obtained data suggest that the mechanism of the observed enhancement of the peripheral blood erythrocyte destruction in acquired hemolytic anemia after Asn ase injection [31] is closely related to the enzyme effect on the erythrocyte immunoglobulin binding in the blood

[1] Supported by The Blood Research Foundation, Washington, D.C.

Materials and Methods

Asn-ase (Crasnitin Bayer) was injected intravenously in a single dose of 500 IU/kg to 2 patients with acquired hemolytic anemia of the IgG and IgA type (patients R T and S A) Determinations of the immunoglobulin content in the serum and on the erythrocyte surface were carried out before the Asn-ase i.v. injection as well as at the 3rd and the 7th day after the enzyme administration

(1) The direct antiglobulin test was made by assaying several antiglobulin sera, i.e., the monovalent sera anti IgG anti IgA and anti IgM produced by Behringwerke AG, the antisera produced by the Institute of Hematology of Budapest the antisera produced by the Transfusiology Station of Cracow The following scale of plus to evaluate the positivity in those tests was used marked (+++) medium (++) slight (+) On the other hand the negativity was indicated with the minus (-)

(2) The serum content of the immunoglobulins IgG IgA and IgM was determined by means of a simple radial diffusion test, according to MANCINI *et al* [23] For these assays, the original Behringwerke immunodiffusion plates for quantitative determination of plasma proteins were used Besides the patient determinations also the IgG IgA and IgM serum content in 25 healthy subjects were determined to take as normal control values.

(3) Immunoglobulins coating erythrocytes were investigated on blood smears by means of the immunofluorescence method using the fluoresce n labelled sheep globulin directed against human immunoglobulin IgG IgM produced by Wellcome (UK) Air dried blood smears were stored at 0–4 °C for several days, until all the samples from the same patient were collected Then the smears were fixed for 15 min in cold absolute aceton and cold absolute ethanol After a short washing in phosphate-buffered isotonic saline at pH 7.2, a small area of the smear was covered with the drop of fluorescent serum and incubated for 45 min at room temperature in wet chamber Thereafter, the smears were rinsed in 3 subsequent changes of phosphate-buffered saline 10 min each embedded in buffered glycerol at pH 9.0 and viewed under fluorescent light of the mercury lamp HBO 50. Suitable pictures were taken under the light of a stronger lamp HBO 200, using the Ilford film EP 4

The following controls for the specificity of the reaction were made blood smears without any fluorescent agent applied smears from the blood of the investigated patients covered with antirabbit fluorescent serum smears from rabbit blood covered with antihuman fluorescent serum Besides, the blood smears from 2 subjects without any detectable hematological disorder were also incubated with antihuman IgG IgM fluorescent serum All the above-mentioned controls did negative results

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Table I Results of the direct antiglobulin test

	Anti globulin sera and patients					
	IgG		IgA		IgM	
	R T	S A	R T	S A	R T	S A
Before the Asn ase administration	+	+++	+++	+++	+	+
3rd day after the Asn ase administration	+	+	+++	+++	–	–
7th day after the Asn ase administration	±	±	++	++	–	–

Table II Content of the immunoglobulins IgG, IgA and IgM in the serum (mg/100 ml)

		Immunoglobulins and patients					
		IgG		IgA		IgM	
		R T	S A	R T	S A	R T	S A
Before the Asn ase *administration*		*1320*	*1400*	*448*	*268*	*258*	*254*
3rd day after the Asn-ase administration		1320	2400	590	436	322	336
7th day after the Asn-ase administration		1420	1840	595	412	310	294
Control values from	means	1117		225		177 7	
25 healthy subjects	ranges	920–1440		100–300		85–234	

after the Asn-ase i v injection, a decrease of the intensity of the antiglobulin test reaction did occur

The content of immunoglobulins IgG, IgA and IgM in the serum The results obtained in the 2 patients are shown in table II Those data show a significant increase of the serum immunoglobulin content in the subsequent days after the Asn-ase administration

The immunoglobulins coating the erythrocytes Before the Asn ase i v administration, the blood smears of the patient S A, with acquired hemolytic anemia, showed a marked fluorescence localized on the surface of the majority of erythrocytes (fig 1a) That erythrocyte fluorescence disappeared 2 h after the Asn ase injection, and fluorescence of

the background of the blood smear (serum) was noted It seems worth mentioning that the fluorescence of the background was more intense in the areas close to erythrocytes The above changes were especially well marked on the 3rd day after the Asn-ase injection (fig 1b), and at the 5th day the recovery of the erythrocyte fluorescence was seen (fig 1c)

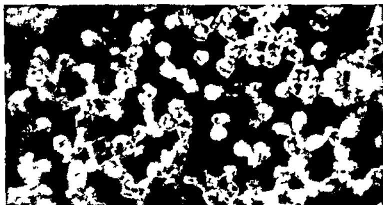
The blood smears of the patient R T showed an erythrocyte fluorescence less marked before the Asn-ase injection, when compared to that of the patient S A The fluorescence of the peripheral zone of erythrocytes was, however, well seen also in this case (fig 2a) Two hours after the Asn-ase injection, the erythrocyte fluorescence did not change significantly, and only slight fluorescence of the background appeared at this time On the contrary, a marked decrease of erythrocyte fluorescence was observed 3 days after the onset of the experiment, analogous to the first case (fig 2b) The erythrocyte fluorescence was back at the initial intensity after 2 weeks (fig 2c)

The blood smears of normal subjects showed no erythrocyte fluorescence (fig 3), but there was a fluorescence located in the background of the erythrocytes (serum)

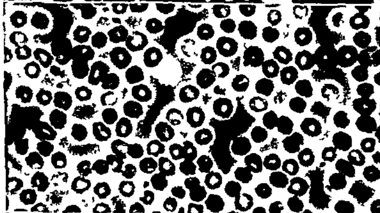
Discussion

It is now definitely proven that Asn-ase has immunological properties First of all, it was shown that this enzyme inhibits lymphocytes blastogenesis in cell culture [4], even at small doses [1] Again, it appeared that the above-mentioned inhibition may occur either when stimulation is made via plant extracts, or via soluble antigens to which the lymphocyte donors were previously immunized, as well as when the lymphocytes are stimulated by using some chemical compounds, and also in the mixed lymphocyte reaction, where allogenic lymphocytes, having different transplantation type antigens, are mutually stimulating [5–7] The inhibition of lymphocyte blastogenesis is not due to a toxic effect caused by the enzyme preparation, as it was suggested by DARTNALL and BAIKIE [12], but it does really depend on the L-asparagine de-

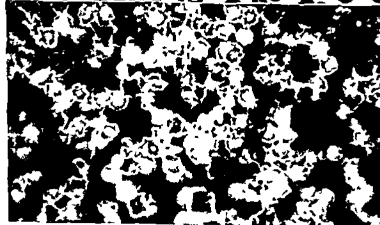
Fig 1 Patient S A, with acquired hemolytic anemia *a* Marked fluorescence on the surface of many erythrocytes before the Asn ase administration *b* Already 2 h after a single i v Asn ase injection of 500 IU/kg there was fluorescence in the *erythrocyte background (serum) whereas the fluorescence of the erythrocyte sur* faces had disappeared This finding was more marked at the 3rd day after the Asn ase injection *c* The recovery of the erythrocyte fluorescence was noted at the 5th day after the Asn ase injection



a



b



c

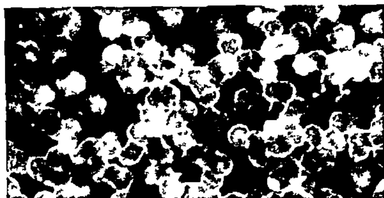
pletion [3, 9, 32] It was also demonstrated that lymphocytes, both tumor [26] and normal [28], do not posses any asparagine-synthetase

Then, it was observed that Asn-ase may (1) delay the graft rejection [8, 29, 30], (2) depress the graft-versus host-reaction [15], (3) depress the hemagglutinin titers in mice, when immunized either with human donor erythrocytes [27] or sheep erythrocytes [11], (4) depress the antitransferrin formation in mice following immunization with bovine transferrin [20], (5) inhibit the hemolytic plaque formation tested by the Jerne technique [21, 22] Furthermore, it did result that high doses of Asn-ase, such as 1,000 IU/kg/day or more, may also suppress the antibody formation and the delayed hypersensitivity [17, 21], may inhibit the experimental allergic encephalomyelitis in rats [18], as well as exert a preventive effect on the adjuvant-induced polyarthritis in rats [22], and cause remissions also in man-allergic disorders [14]

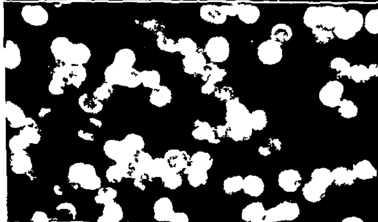
On the contrary, Asn-ase at small doses (100 IU/kg/day, or less) is antigenic, and causes formation of precipiting and complement-binding antibodies [16, 24] which have been crystallized by DE BARBIERI *et al* [13] *Again, it was shown that Asn-ase may stimulate the germinal centers* of the lymphatic follicles, and plasma cell formation [2] In the serum of patients undergoing Asn-ase treatment, an increase of the IgG, IgA, and IgM immunoglobulins was also observed [10, 25] In the study reported here, we too did observe an increase of the serum immunoglobulin content in our patients with acquired hemolytic anemia submitted to a single i v Asn-ase injection of 500 IU/kg, but we did also observe the simultaneous release of immunoglobulins attached to the erythrocyte surfaces In general, the results of antiglobulin tests were in accordance with the above observations Henceforth, it seems highly probable that the increase of the immunoglobulin contents in the serum was due to their release from erythrocytes The mechanism of the Asn-ase action on the binding between the antigenic determinants of erythrocytes surface and the immunoglobulins is not clear In our opinion, the direct effect of Asn-ase on this binding is possible, since the immunoglobulin release

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Fig 2 Patient R T with acquired hemolytic anemia *a* Fluorescence on the erythrocyte surfaces before the Asn ase injection The erythrocyte fluorescence intensity in this patient is less marked when compared to the intensity observed in the patient S A (fig 1) *b* Intense decrease of the erythrocyte fluorescence observed on the 3rd day after the Asn ase administration *c* The erythrocyte fluorescence intensity is back to the original extent 2 weeks after the experiment onset


a


b


c

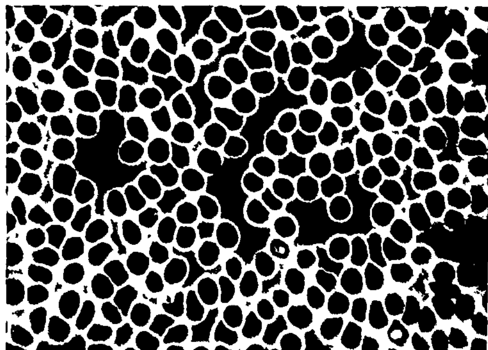


Fig 3 In healthy subject blood smears, only fluorescence of the erythrocyte background (serum) was seen whereas the erythrocyte surfaces did never exhibit fluorescence

into the background of the blood smears was seen as soon as 2 h after the enzyme injection It seems, therefore, improbable that the observed effect was due to the Asn-ase immunodepressive activity In other words, these results seem to suggest that, at least in some conditions such as in acquired hemolytic anemia, the mechanism of the increase of the serum immunoglobulin content after the Asn-ase injection may be different from that admitted in other disorders It also derives that the release of immunoglobulins from the target cells in autoimmune diseases, or other disorders with positive antiglobulin tests, offers a possible way to increase the immunoglobulin content in the serum

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Acta haemat 47 81–88 (1972)

'Prophylactic' Treatment of Meningeal Leukemia in Children by Intrathecal Methotrexate [1]

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Abstract The survival period of children suffering from leukemia could be prolonged by using the new antileukemic agents On the other hand the frequency of central nervous system involvement as a complication of this disease was very high, i e 10 of 19 cases Six showed signs of meningeal leukemia while they were in hematologic remission and the other 4 were within acute stage Nine children were given methotrexate intrathecally as a prophylactic measure at the time when diagnosis of leukemia was confirmed and did not suffer from this complication during follow up to 6–13 months From these data we conclude that the regular administration of methotrexate intrathecally to leukemic patients as a prophylactic measure with routine spinal fluid examinations is very useful in preventing or in reducing the central nervous system involvement

Key Words
Children leukemia
Leukemia treatment
Meningeal leukemia
Methotrexate

The new antileukemic agents such as vincristine, methotrexate, purinethol, daunoblastine, etc , have prolonged the lives of children suffering from leukemia by several months or even several years On the other hand, there has been an increase in meningeal leukemia This is probably because more patients are living long enough to develop detectable meningeal involvement

Leukemia is a systemic disease and leukemic infiltration can spread throughout the body One of the most dreaded complications is central nervous system involvement According to WHITESIDE [1], PIERCE [2], and NIERI [3] the development of this complication might be due to the failure of drugs to pass across the blood-brain barrier Various opinions

[1] This paper has been presented in the 2nd Meeting of the Asian Pacific Division of the International Society of Haematology, Melbourne, May 24–28 1971

Table I Patients with

Case No	Age, years Sex		Hb g%	Leuco / mm³	Thromb / mm³	Differential count, %						
						eos	baso	stab	segment	lympho	mono	*pathologic* cells
1	3 0	♂	10 0	80 000	340 000	2	–	–	35	56	5	+
2	4 0	♂	6 8	8 400	357,000	1	1	–	67	27	4	–
3	3 6	♀	8 5	16 200	172,000	1	–	2	19	78	–	+
4	4 0	♂	13 5	5,900	338,000	–	–	–	45	54	1	–
5	8 0	♀	11 0	5 800	282,000	4	–	–	59	36	1	–
6	7 0	♂	10 6	10,800	225 000	–	3	–	50	44	3	–
7	5 6	♂	12 0	8 000	282 000	5	–	1	35	54	5	–
8	6 0	♂	5 5	4 200	147,000	–	–	1	14	80	5	–
9	4 0	♀	3 0	25,600	0	–	–	–	2	98	–	+
10	7 0	♂	10 0	5,400	182 000	3	–	3	40	49	5	–

¹ ALL, acute lymphocytic leukemia

have been put forward about the occurrence of leukemic cells in the central nervous system According to SULLIVAN [4] the increment of the cells in the cerebrospinal fluid is caused by the infiltration of leukemic cells into the meninges KAPPERS [5] assumed that the chorioid plexus of human embryo has the ability to produce blood cells He found that an i v injection of methyl-violet into the guinea pig caused proliferation of histiocytes, lymphocytes, granulocytes and eosinophils in the stroma of the chorioid plexus The cytogenetic examination of the spinal fluid of 9 children with acute leukemia, by MASTRANGELO *et al* [6], revealed an identity or similarity of the chromosomal constitution of the leukemic cell populations *in the cerebrospinal fluid and in the bone marrow* The

meningeal leukemia

Bone marrow picture[1]	Spleen	Neurologic signs	Spinal fluid findings: nonne	Pandy	cell count /mm³	Time of onset of meningeal leukemia, month(s)	Duration of treatment week(s)
ALL in relapse	S_1	convulsion paresis of facial nerve	+	+	823/3	12	2
ALL in remission	S_0	visual disturbances	+	+	3 675/3	8	4
ALL in relapse	S_0	headache and dizziness	+	+	726/3	6	2
ALL in remission	S_0	headache vomiting	+	+	4,232/3	8	8
ALL in remission	S_0	symptom free	+	+	784/3	8	4
ALL in remission	S_0	visual disturbances	+	+	826/3	7	4
ALL in remission	S_0	headache dizziness	+	+	3 712/3	7	1
ALL in relapse	S_1	dizziness	+	+	3 160/3	12	2
ALL	S_0	dizziness	+	+	317/3	1	1 day
ALL in remission	S_0	paresis of facial nerve	+	+	857/3	3	2

findings are presented as direct evidence for the metastatic nature of meningeal leukemia

LEIDLER and RUSSEL [7] reported that on autopsy examination almost 56% of their leukemic patients had developed central nervous system involvement but not all of these patients had shown clinical neurologic disturbances HYMAN *et al* [8] reported that only 26–30% of their cases showed signs of such involvement while PIERCE [2] *found only* 11 2%. Those syndromes are caused by the infiltration of the leukemic cells into the choroid plexus, arachnoid or other parts of the *meninges* and even the brain tissue There are 2 types of central *nervous system* leukemia, i e, the meningeal type and the *cerebral type [10] The for-*

mer is the more common. The signs and symptoms of this type are not very severe and easy to relieve by giving the intrathecal drugs. In the cerebral type, the symptoms and signs are more serious and the patient *is not likely to survive for long. Generally the clinical symptoms such as* dizziness, irritability, headache, vomiting and visual disturbances are caused by the increase of intracranial pressure.

The purpose of this paper is to present our experience in handling leukemic patients during the last 2 years at the Department of Child Health, Medical School, University of Indonesia, Djakarta.

Materials and Methods

Nineteen leukemic patients were admitted to our Department of Child Health, Medical School, University of Indonesia, Djakarta, from the beginning of 1969 to the end of 1970. There were 12 boys and 7 girls varying in age from 3 to 11 years Peripheral blood examination, bone marrow puncture and spinal fluid examination were done routinely. The diagnosis of all cases was confirmed by bone marrow examination All children were treated by the combination of vincristine (0 03–0 05 mg/kg body weight, i v, once a week), methotrexate (30 mg/m^2 body surface, orally, twice a week), 6 mercaptopurine (2 5–5 mg/kg body weight, orally, everyday) and prednisone (2–4 mg/kg body weight, orally, everyday) Blood transfusion was given if necessary If remission was achieved, all drugs were discontinued except methotrexate which was continued at the same dosage as a maintenance treatment The combination was repeated every 3–4 months for 10 days as a re-induction treatment

These 19 patients were divided into 2 groups Group 1 consisted of 10 children (table I) In this group the lumbar puncture was done when the children showed signs of increased intracranial pressure such as headache, vomiting, etc Intrathecal methotrexate was given simultaneously with the lumbar puncture In group 2 (table II) lumbar puncture was performed and methotrexate administered intrathecally as soon as the diagnosis of leukemia was established If there was no abnormality in the spinal fluid, the injection was repeated 1 week later and then continued every 3–4 months even though the patient was symptom free Patients of either group who showed abnormality in the spinal fluid were given intrathecal methotrexate twice a week until the cells in the spinal fluid returned to normal levels (less than 10/mm^3) In cases No 1, 2 and 4 of group 1, radiotherapy was given in addition to the intrathecal injections of methotrexate.

Results and Discussion

In the 1st group (table I) meningeal involvement developed from 1 to 12 months after the diagnosis of leukemia was confirmed Six chil-

Table II. Patients who got prophylactic intrathecal methotrexate at the day of admission

Case No	Age years	Sex	Hb g%	Leuco/mm³	Thromb/mm³	Differential count %								Bone marrow[1]	Spinal fluid findings on admission	Spinal fluid findings during follow-up	Duration of follow-up (months)
						eos	baso	myelo	stab	segment	lympho	mono	pathol. cells				
1	11	♂	6.8	4 500	334 000	1	1		3	12	84			ALL	normal	normal	12
2	1.6	♀	5.5	4 500	235 000	3				36	59	2	+	ALL	normal	normal	13
3	4.6	♂	5.6	24,200	90 000	2			1	24	70	3		ALL	normal	normal	8
4	11	♀	7.4	6 400	44 000	2		4	1	10	81	2	–	AEL	normal	normal	12
5	11	♀	3.4	13 200	52 000	5			1	40	52	2	+	ALL	normal	normal	1
6	9	♀	3.9	1 000	15 000	0		1	3	51	35	9	–	AEL	normal	normal	6
7	5	♂	5.6	13 200	11 000				3	5	85	4	–	AEL	normal	normal	1
8	8	♂	8.0	61 900	62 000	1			3	5	93	1	+	AEL	normal	normal	12
9	0.6	♂	2.0	17 000	8 200					12	85	3		AEL	normal	normal	6

[1] ALL, acute lymphocytic leukemia, AEL, acute erythroleukemia

mer is the more common The signs and symptoms of this type are no very severe and easy to relieve by giving the intrathecal drugs In tl cerebral type, the symptoms and signs are more serious and the pati is not likely to survive for long Generally the clinical symptoms such dizziness, irritability, headache, vomiting and visual disturbances caused by the increase of intracranial pressure

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Results and Discussion

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ment of central nervous system involvement Because of the difficulty of measuring the level of this drug in the spinal fluid it is still problematical that the injection of methotrexate by this method is adequate We used it in this study because the average period of hematologic remission in our material was 3 months and the early stage of meningeal leukemia could easily be overcome It does appear that regular administrations of methotrexate intrathecally as a prophylactic procedure may delay the possibility of meningeal leukemia

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Acta haemat. 47: 89–97 (1972)

Eine einfache Methode der Knochenmarkkurzzeitkultur

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Abstract. A rapid method of culturing bone marrow fragments in liquid medium under standard conditions is described. The suitability of various media has been investigated, and these are characterized according to their morphological features, cell counts and determination of the mitotic index.

Key Words:
Bone marrow culture
Granulocytopoiesis *in vitro*
Erythropoiesis *in vitro*

Zahlreiche klinische Fragestellungen machen Knochenmarkkulturen erforderlich, die es ermöglichen, alles zu untersuchende Gewebe gleichzeitig unter gleichen, jederzeit veränderlichen Bedingungen zu kultivieren. Dieses Knochenmark sollte alle Zellsysteme der Hämopoese in ihrem natürlichen Verband und Mengenverhältnis enthalten. Diese Voraussetzungen sind dann erfüllt, wenn Knochenmarkbröckel, wie sie bei der Knochenmarkpunktion gewonnen werden, in flüssigem Kulturmedium gemeinsam in einer Kulturflasche gezüchtet werden. Hierbei darf eine bestimmte Grösse der Knochenmarkbröckel nicht überschritten werden, um eine Ernährung durch Diffusion nicht zu gefährden.

Schon seit Beginn des Jahrhunderts sind Kulturen von Knochenmarkgewebe bekannt [3, 5–7, 11, 16]. Doch wurde hierbei koaguliertes Plasma als Kulturmedium benutzt und für jedes Gewebeteilchen eine gesonderte Kultur angelegt. Demgegenüber werden bei den Suspensionsmethoden einzelne, nicht im Verband liegende Knochenmarkzellen in flüssigem Kulturmedium suspendiert, wobei es sich meist um menschliches Serum mit wachstumstimulierenden Zusätzen handelt [1, 2, 4, 8–14, 16–22]. Diese Methoden haben den grossen Vorteil, dass das Wachstum der Kulturen nach den durchschnittlichen Zellzahlen beurteilt werden kann, jedoch den grossen Nachteil, dass die frei beweglichen

Knochenmarkzellen nicht reprasentativ fur das gesamte Knochenmark sind, sondern nur Zellstudien ermoglichen. Hierher gehoren auch die Agar-Kulturen, bei denen auf Agar-Nahrboden Knochenmarkzellen einzeln – also nicht im Verband – gezuchtet werden.

Die hier darzustellende Kulturmethode bietet durch Kombination von Vorteilen beider Methoden die Voraussetzungen, wie sie zur Beantwortung klinischer Fragestellungen zu fordern sind, insbesondere zur Testung verschiedener Pharmaka bzw. zur Prufung der Ansprechbarkeit erkrankten Knochenmarkgewebes auf verschiedene Pharmaka.

Methode

Die Knochenmarkaspiration erfolgt aus dem Sternum oder Beckenkamm in eine 0,5 ml EDTA (Di-Na der Aethylendiamintetraessigsaure) und Titriplex III* (Fa. Merck) enthaltende 20 ml Einmalspritze. Mittels eines sterilen Deckglases werden die am Boden haftenden Knochenmarkbrockel abgehoben und mit vorgewarmtem Kulturmedium von der Kante des Deckglases in die ebenfalls vorgewarmte sterile Kulturflasche abgespult. Die Menge des Kulturmediums wird mit 2 bzw. 4 ml konstant gehalten. Es empfiehlt sich, das Kulturmedium und die darin schwimmenden Knochenmarkgewebeteilchen in eine 5 ml Einmalspritze aufzusaugen und durch eine Einerkanule in eine zweite Kulturflasche zu spritzen. Die Kulturflasche muss hierfur nicht geoffnet werden. Man erhalt hierdurch Gewebeteilchen mit einer dem Lumen der Nadel entsprechenden Maximalgrosse.

Die Öffnung der Kulturflasche wird jeweils mit einem Bunsenbrenner kurz abgeflammt, bevor der Deckel, der in seiner Mitte eine gummiverschlossene runde Perforation tragt, aufgeschraubt wird. Die Kultur wird im Brutschrank bei 37 °C bebrutet. Nach gewunschten Kulturzeiten – im vorliegenden Material bei 2, 4, 8, 16, 24 und 32 h – werden nach Öffnen der Kulturflaschen mit sterilen Pasteurpipetten im Kulturmedium schwimmende Gewebeteilchen entnommen, auf schrag stehendem Objekttrager von ihrer Flussigkeit getrennt, mit einem sterilen Deckglas abgehoben und auf Objekttragern ausgestrichen. Die Ausstriche werden den jeweiligen Farbemethoden unterzogen.

Das Kulturmedium wird nach 8–10 h zur Halfte ausgewechselt, indem durch die Perforation des Deckels 2 ml Kulturmedium mit einer 12er Kanule tragenden Spritze abgesaugt und die gleiche Menge neues vorgewarmtes Kulturmedium zugesetzt wird.

Der Kultur konnen gleich bei Beginn oder nach beliebiger Kulturzeit wasserlosliche Stoffe zugesetzt werden, wie ^{3}H-Thymidin und ^{3}H-Cytidin bei Isotopenuntersuchungen oder verschiedene Pharmaka, deren Einfluss auf das Knochenmarkgewebe untersucht werden soll.

Befunde

Um diese Kulturmethode angeben zu konnen, wurde in über 100 Kulturen die Frage gepruft, ob und wie lange Knochenmarkbrockel in

flüssigen Kulturmedium bleiben können, wie das Kulturmedium beschaffen sein muss und welche Veränderungen des Knochenmarkgewebes durch die besonderen, stets gleichbleibenden Kulturbedingungen verursacht sind. In 33 Kulturen wurde die Kultur sowohl nach 2, 4, 8, 18, 24 und 32 h Kulturdauer an Knochenmarkausstrichen jeweils 500–1000 Zellen ausgezählt und Myelogramme angelegt, ferner der Mitoseindex an je 1000–3000 Erythroblasten bestimmt.

Lebensdauer. Hierbei zeigte sich, dass diese Kulturen in einer Beobachtungszeit von 24–32 h nach morphologischen Kriterien und Mitoseindexbestimmungen dem »Zustand in vivo« vergleichbar sind.

Kulturmedium. Von verschiedenen getesteten Kulturmedien eignet sich das Serum des Knochenmarkspenders, Serum aus Aderlassen von Patienten, die an einer Polyglobulie oder Polycythaemia leiden, Serum von Normalpersonen bei Blutgruppengleichheit oder bei Vorliegen der Blutgruppe AB. Zusatz von Hanksscher Lösung im Verhältnis 1:1 hat keinen nachteiligen Einfluss auf die Güte der Kulturen in einer Kulturzeit von 32 h. Fetales Kalbserum kann zugesetzt werden, weist jedoch keinen sicheren stimulierenden Einfluss auf; als alleiniges Kulturmedium ist es jedoch ungeeignet.

Grösse der Knochenmarkbröckel. Die Grösse der Knochenmarkbröckel, wie sie bei der Aspiration gewonnen werden, ist abhängig von der Erkrankung und dem Funktionszustand des Knochenmarkes. Diese Gewebeteilchen lassen sich auf eine Maximalgrösse reduzieren, indem das die Bröckel enthaltende Kulturmedium durch eine Einerkanüle gespritzt wird. Die Bröckel werden hierdurch zerkleinert, ohne dass den einzelnen Zellen Schaden zugefügt wird.

Eigenarten der Kulturmethode

Die im folgenden dargestellten Veränderungen sind allen Kulturen gemeinsam, unabhängig von der Erkrankung des Knochenmarkes.

Makroskopisch. Hierbei fällt auf, dass im Verlauf der Kultur sich das Kulturmedium etwas trübt und die durch Zusatz von Hankscher Lösung bedingte rötliche Farbe abblasst, was auf eine Änderung des pH-Wertes hinweist. Die Knochenmarkbröckel nehmen schon nach wenigen Stunden an Grösse zu, was durch Wassereinlagerung in das Interstitium bedingt ist. Je nach Fettgehalt schwimmen sie auf der Flüssigkeit, schweben oder lagern sich am Boden ab, ohne miteinander zu verbacken. Mit zunehmender Kulturzeit über 24 h zeigte sich das Knochenmarkgewebe derber und faseriger.

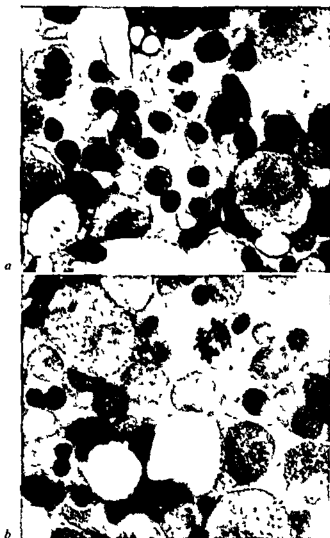


Abb 1 Zellbild des Knochenmarkes in der Kultur *a* Nach 8 h *b* Nach 18 h (Renale Anämie)

Mikroskopisch Als Ausdruck der veränderten Lebensbedingungen und des gestörten Gleichgewichtes von Zellwachstum und Zelluntergang in der Kultur gegenuber den «Bedingungen *in situ*» finden sich in allen Kulturen Veranderungen Ab der 8, vereinzelt schon ab der 4 Kulturstunde finden sich kleine Vakuolen im Zytoplasma der Zellen der Erythropoese und der Granulopoese verschiedener Reifungsstufen, die in Abhangigkeit von der Kulturdauer an Grosse und Zahl zunehmen Ver-

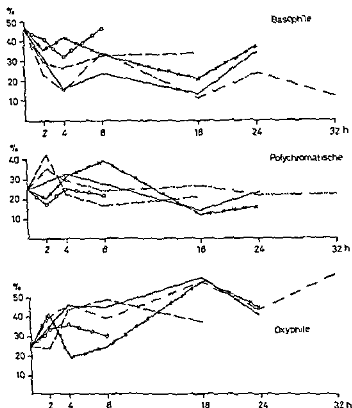
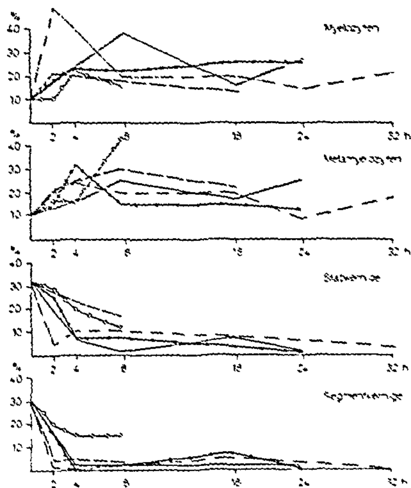


Abb 2 Erythropoese in der Kultur bei verschiedenen Kulturmed en *Renale Anämie* -.-.- *Polyglobulieserum*, - - - Hanks und Polyglobuli*eserum*, x-x-x-x *Polycythämie*-serum, o-o-o-o fetales Kälberserum, —— Hanks und *Polycythämieserum*

einzelt finden sich auch solche Vakuolen in Zellkernen Die Zellkerne erscheinen zunachst weich, spater verwaschen und homogen, mitunter sieht man eine Auflosung von Kern- und Zellmembran Solche degenerierende Knochenmarkzellen haufen sich mit zunehmender Kulturdauer Der Mitoseindex kann unter Kulturbedingungen ansteigen oder abfallen gegenuber dem Ausgangswert in Abhangigkeit von der Erkrankung des Knochenmarkes

Die einzelnen Zellsysteme weisen charakteristische Veranderungen auf


%
40
30
20
10
Myelozyten
2
4
8
16
24
32 h
Metamyelozyten
Stabkernige
Segmentkernige

Durchschnitt erfolgt ein Abfall von 25% auf 3–5% Dieses Niveau wird bis Kulturende beibehalten In einigen Fällen steigt die Zahl der reifen Granulozyten gegen Ende der Kulturen wieder an Demgegenuber zeigen die Metamyelozyten kein abweichendes Verhalten Die reifen Myelozyten verhalten sich den stab- und segmentkernigen gegenuber entgegengesetzt Die Durchschnittswerte verdoppeln sich bis Kulturende In einigen Kulturen fallen die Werte nach der 16 Stunde wieder ab Die Eosinophilen verhalten sich ahnlich wie die reifen Myelozyten Doch erfolgt der Anstieg gegenuber den reifen Myelozyten verzogert und nicht so ausgepragt Die Durchschnittswerte der Eosinophilen liegen bei Beginn der Kultur bei 9% und steigen im Verlauf der Kultur auf 15,1% an Diese Zunahme der Eosinophilen muss ebenso wie die Zunahme der reifen Myelozyten im Zusammenhang mit der Abnahme der reifen Granulozyten gesehen werden, da es sich um Relativwerte handelt

Abhangigkeit vom Kulturmedium

Es besteht eine Abhangigkeit des kultivierten Knochenmarkgewebes vom Kulturmedium insofern als in ungeeignetem Kulturmedium die Lebensdauer verkurzt ist und die degenerativen Veranderungen fruher einsetzen Entsprechend bleiben die Mitosen fruher aus Nach diesen Kriterien ist das Serum des Knochenmarkspenders und Serum von Normal personen bei Blutgruppengleichheit in gleicher Weise geeignet, sofern keine besonderen Erkrankungen mit gesteigerter oder verminderter Re generation bestehen Serum von Patienten, die an einer Polyglobulie leiden, eignet sich ebenfalls als Kulturmedium und wirkt besonders anregend auf die Erythropoese, verglichen mit Serum von Normalpersonen Eine Verdunnung des jeweiligen Serums mit Hanksscher Losung im Verhaltnis 1 1 hat keine nachteiligen Auswirkungen auf die Kulturen in der Beobachtungszeit von 32 h Zusatz von fetalem Kalberserum zeigt keine stimulierende Wirkung Fetales Kalberserum allein ist ungeeignet Das Kulturmedium muss alle 8–10 h erneuert werden, da sonst die Lebensdauer der Kulturen zuruckgeht und degenerative Veranderungen ver fruht einsetzen

Zusammenfassung

Es wird berichtet uber eine Kurzzeitkulturmethode von Knochenmarkbrockeln die in flussigem Kulturmedium unter gleichen Bedingungen kultiviert werden kon nen Die Eignung verschiedener Kulturmedien wurde untersucht und die Eigenar

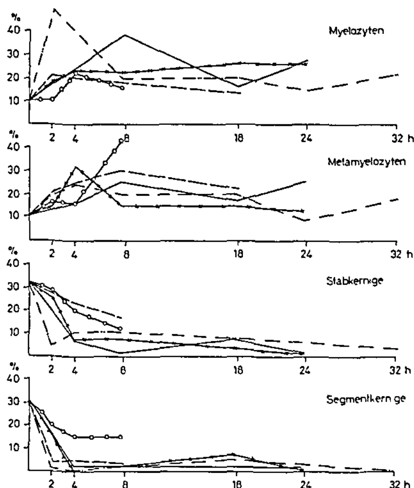


Abb 3 Granulopoese in der Kultur bei verschiedenen Kulturmedien Renale Anämie ---- Polyglobulieserum, - - - Hanks und Polyglobulieserum x-x x-x Polycythämieserum, o-o-o-o fetales Kalberserum —— Hanks und Polycythämieserum

Erythropoese (Abb 2) In der Erythropoese findet sich mit zunehmender Kulturdauer eine Verschiebung zu den reifen oxyphilen Erythroblasten Davon sind die Proerythroblasten ausgenommen, die bis zum Kulturende in gleicher Haufigkeit anzutreffen sind Die Anzahl absterbender Zellen nimmt mit der Kulturdauer zu Der Mitoseindex steigt an oder fallt ab in Abhangigkeit von der Art der Erkrankung Man beobachtet zahlreiche Erythroblasten mit einfach oder mehrfach segmentierten Kernen mit einem Maximum nach 8 h

Granulopoese (Abb 3) Sehr eindrucksvoll ist der Verlust der stabkernigen und segmentkernigen Granulozyten in den ersten 4 h Im

Durchschnitt erfolgt ein Abfall von 25% auf 3–5%. Dieses Niveau wird bis Kulturende beibehalten. In einigen Fallen steigt die Zahl der reifen Granulozyten gegen Ende der Kulturen wieder an. Demgegenuber zeigen die Metamyelozyten kein abweichendes Verhalten. Die reifen Myelozyten verhalten sich den stab- und segmentkernigen gegenuber entgegengesetzt. Die Durchschnittswerte verdoppeln sich bis Kulturende. In einigen Kulturen fallen die Werte nach der 16. Stunde wieder ab. Die Eosinophilen verhalten sich ahnlich wie die reifen Myelozyten. Doch erfolgt der Anstieg gegenuber den reifen Myelozyten verzogert und nicht so ausgepragt. Die Durchschnittswerte der Eosinophilen liegen bei Beginn der Kultur bei 9% und steigen im Verlauf der Kultur auf 15,1% an. Diese Zunahme der Eosinophilen muss ebenso wie die Zunahme der reifen Myelozyten im Zusammenhang mit der Abnahme der reifen Granulozyten gesehen werden, da es sich um Relativwerte handelt.

Abhangigkeit vom Kulturmedium

Es besteht eine Abhangigkeit des kultivierten Knochenmarkgewebes vom Kulturmedium insofern als in ungeeignetem Kulturmedium die Lebensdauer verkurzt ist und die degenerativen Veranderungen fruher ein setzen. Entsprechend bleiben die Mitosen fruher aus. Nach diesen Kriterien ist das Serum des Knochenmarkspenders und Serum von Normalpersonen bei Blutgruppengleichheit in gleicher Weise geeignet, sofern keine besonderen Erkrankungen mit gesteigerter oder verminderter Regeneration bestehen. Serum von Patienten, die an einer Polyglobulie leiden, eignet sich ebenfalls als Kulturmedium und wirkt besonders anregend auf die Erythropoese, verglichen mit Serum von Normalpersonen. Eine Verdunnung des jeweiligen Serums mit Hanksscher Losung im Verhaltnis 1:1 hat keine nachteiligen Auswirkungen auf die Kulturen in der Beobachtungszeit von 32 h. Zusatz von fetalem Kalberserum zeigt keine stimulierende Wirkung. Fetales Kalberserum allein ist ungeeignet. Das Kulturmedium muss alle 8–10 h erneuert werden, da sonst die Lebensdauer der Kulturen zuruckgeht und degenerative Veranderungen verfruht einsetzen.

Zusammenfassung

Es wird berichtet uber eine Kurzzeitkulturmethode von Knochenmarkbrockeln die in flussigem Kulturmed um unter gleichen Bedingungen kultiviert werden konnen. Die Eignung verschiedener Kulturmedien wurde untersucht und die Eigenar

ten der Kultur nach morphologischen Kriterien, Zellzahlungen und Mitoseindexbestimmungen beschrieben

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The Urinary Excretion of Haemoglobin in the Isolated Normothermic-Perfused Dog Kidney

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Abstract The isolated kidney handles haemoglobin in the same manner as the kidney *in vivo* There is no excretion of haemoglobin when the haemoglobin binding capacity is not saturated When this is done and a free plasma haemoglobin level exists, the clearance of free haemoglobin had an average value of 3 24±2 92 ml/min The glomerular clearance ratio of haemoglobin averaged 0 10±0 06 The calculated rate of tubular reabsorption of haemoglobin was 2 06±3 12 mg/min The graphic relationship between urinary excretion and plasma levels of free haemoglobin was linear The theoretical plasma level above which haemoglobinuria occurred was 64 0±35 2 mg% These last two data are more suggestive of tubular reabsorptive activity than the calculated values

Key Words
Dog kidney
Haemoglobinaemia
Haemoglobinuria
Haemolysis
Haptoglobin
Plasma haemoglobin

The plasma haemoglobin renal threshold results from 2 factors Primarily much of the plasma haemoglobin is bound to haptoglobin which has a molecular weight of about 85,000 with an electrophoretical mobility of an α_2-globulin fraction [12], which is not filterable by the renal glomerulus Not until the haemoglobin-binding capacity of the plasma, normally equivalent to about 75 mg% [VAN DER STOCK, unpublished observation], is exceeded and free haemoglobin is present, can significant filtration of haemoglobin begin [13], primarily as α-β-dimers [5] Filtered haemoglobin is extensively taken up by the cells of the proximal tubule [10] Filtered haemoglobin must exceed reabsorptive capacity, or Tm, before significant excretion commences Tm in the dog is about 3–4 mg/min [13]

The present work was undertaken to study mechanisms of clearance of plasma haemoglobin and to determine the tubular recovery values for

haemoglobin in the isolated perfused dog kidney. Previous studies from our laboratory [9] confirmed the observations of others, that the isolated blood perfused dog kidney undergoes a marked haemodynamic and functional alteration during the course of perfusion. We want to know if this isolated kidney with decreased functional capacity was still able to handle haemoglobin in the same manner as the kidney *in vivo* and *in situ* in the dog. The acute haemolysis, produced during isolated kidney perfusion, indicated a need for further information on the mechanisms involved in the clearance of haemoglobin from circulating plasma.

Materials and Methods

We used a modification of the perfusion apparatus of CUYPERS *et al.* [7]. The perfusion circuit and its operations have been discussed in a previous article from our laboratory [8]. In another study [9] we have determined the functional capacity of these isolated kidneys. 20 mongrel dogs weighing no less than 15 kg are selected without preference as to sex or age. For the detailed description of basic experimental conditions we refer to the previous paper [8]. The haemoglobin binding capacity of the serum is determined by a direct colorimetric method [16] with gajacol. The free haemoglobin plasma level is measured by the method of CROSBY and FURTH [6]. The urine haemoglobin concentration is determined by the method of ZIJLSTRA and VAN KAMPEN [21]. Creatinine is measured in the serum by the method of BROD and SIROTA [3] and in the urine by the method of BONSNES and TAUSSKY [2]. In some plasma (from heparinised dogs) the initial level of free or protein-bound haemoglobin was low, in other plasma there was more initial haemolysis by defibrination of the blood. To some other plasma, we added a quantity of free haemoglobin to raise the initial plasma haemoglobin level. However, in all plasma, there was a progressive rise of protein bound or free haemoglobin by haemolysis produced by mechanical stirring of the perfusion apparatus. The rate of haemolysis will soon appear in another study of our laboratory [9]. Therefore, in certain perfusions at the very beginning of the experiment there was an excretion of haemoglobin in the urine. In other perfusions, the concentration of protein bound haemoglobin rose progressively until a level was attained at which additional binding did not occur and haemoglobin appeared in plasma in the free unbound state, and was also excreted in the urine, only a few hours after the beginning or even at the end of the perfusion.

Results

Excretion of Haemoglobin in the Urine as Long as the Haptoglobin was not Completely Saturated with Haemoglobin

In dogs *in vivo* haemoglobin never appeared in the urine until the

Table I Average excretion of haemoglobin (Hb) in the urine by 10 isolated perfused dog kidneys, when the plasma haemoglobin binding capacity is not saturated with haemoglobin

Experiment No	Serum Hb binding capacity, mg %		Average value of Hb in urine		Perfusion time min
	beginning	end	mg %	mg/min	
18	210	140	0 5	0 00	240
19	48	13	0 6	0 00	90
20	30	13	0 2	0 02	30
21	30	18	0 3	0 01	60
22	93	13	0 1	0 01	30
23	342	132	1 4	0 00	30
24	327	77	4 5	0 04	60
33	42	4	1 1	0 00	60
34	198	18	0 3	0 00	780
35	140	0	1 4	0 00	30
Average	146	44	1 0	0 01	
±SD	±119	± 53	±1 3	±0 01	

plasma haemoglobin concentration exceeds the haptoglobin level [14] Haemoglobin appeared in the urine after the haptoglobin level was significantly exceeded rather than at the first appearance of free haemoglobin in the plasma, in Lathem's work [14] the measured amount of haemoglobin present in excess of the haptoglobin level averaged 27 mg% and ranged up to 60 mg% In table I, we summarise the results of the excretion of haemoglobin in the urine in 10 isolated perfused kidneys, in which the haemoglobin (Hb)-binding capacity was not saturated with haemoglobin

The plasma haemoglobin-binding capacity decreased on an average from 146 ± 119 mg/100 ml to 44 ± 53 mg/100 ml The individual average haemoglobin concentrations in the urine varied from 0 2 to 4 5 mg/100 ml, which corresponded to an excretion of 0 00 to 0 04 mg/min During the course of the perfusion there was little variation in the concentration or in the amount of haemoglobin excreted per unit of time in the individual experiments

Although we cannot avoid a little haemoglobin in the urine we think that the haemoglobin was not filtered by the kidney, but resulted from bleeding into the

renal tract, perhaps by trauma in cannulating the ureter. It must be taken in account that with this method, there are great deviations in the haemoglobin values determined at these low concentrations. Since the urines were diluted, haemolysis of erythrocytes may have occurred with release of haemoglobin. Centrifugation of these urines resulted in a clear urine and microscopic examination of the sediment showed erythrocytes or erythrocyte debris. So there was no excretion of haemoglobin in the urine as long as the plasma haemoglobin binding capacity was not saturated with haemoglobin.

Excretion of Haemoglobin in the Urine when the Plasma Haptoglobin was Completely Saturated with Haemoglobin

It was necessary to distinguish the results of haemoglobin excretion in rising and diminishing urine flow.

Excretion of haemoglobin in rising urine flow. This was studied in 13 experiments. The urine flow rose from 1.18 ± 1.39 ml/min (average ± SD) to 5.43 ± 2.26 ml/min. At the same time the free plasma haemoglobin concentration increased from 77 ± 45 mg% (average ± SD) to 153 ± 100 mg% (average ± SD). When a significant degree of haemoglobinuria is present, change in excretion rate is frequently a linear function of change in plasma concentration [20]. The relationship $\Delta U_{free\ Hb} \cdot V / \Delta P_{free\ Hb}$, has the dimensions of a clearance. Expressed as a fraction of concomitant glomerular filtration rate measured by the clearance of creatinine (C_{Cr}) this fraction has been *variously* termed 'filterability' permeability coefficient' or 'glomerular clearance ratio' (GCR) of Hb [20].

From the relationship between changing concentration of unbound haemoglobin in the plasma ($\Delta P_{free\ Hb}$) and urinary excretion rate ($\Delta U_{free\ Hb} \cdot V$) an estimate of the glomerular clearance of haemoglobin ($C_{free\ Hb}$) may be obtained. As defined by LATHEM [14] glomerular clearance of free haemoglobin $= \Delta U_{free\ Hb} \cdot V / \Delta P_{free\ Hb}$.

The rate of tubular reabsorption of haemoglobin ($T_{free\ Hb}$) was calculated as the difference between the filtered load and the urinary excretion rate for each collection period.

Filtered load mg/min $= C_{free\ Hb} \times P_{free\ Hb}$

T_{Hb} mg/min $= [C_{free\ Hb} \times P_{free\ Hb}] - U_{free\ Hb} \cdot V$

The average individual results of 13 perfusion experiments are listed in table II.

When corrected or calculated for 2 kidneys the glomerular clearance of free haemoglobin varied in the group of 13 *kidneys from* 0.82 to 11.04 and averaged 3.24 ± 2.92 ml/min. The *ratio* $C_{free\ Hb}/C_{Cr}$, tubular

Table II Values, averages and standard deviations of $P_{free\ Hb}$, $C_{free\ Hb}$, $C_{free\ Hb}/C_{cr}$ filtered load, $U_{free\ Hb}$ V and $T_{free\ Hb}$ in 13 isolated perfusions of canine kidneys with rising urine flow

Experiment No	Average $P_{free\ Hb}$, mg%	$C_{free\ Hb}$, ml/min	$C_{free\ Hb}/C_{cr}$	Filtered load, mg/min	$U_{free\ Hb}V$, mg/min	$T_{free\ Hb}$ mg/min
17	129	5 52	0 184	7 13	1 38	5 74
19	42	1 77	0 071	0 75	0 21	0 54
24	63	1 90	0 166	1 19	0 99	0 20
27	96	3 71	0 117	3 56	1 01	2 55
28	61	0 77	0 148	0 47	0 20	0 27
29	123	1 11	0 063	1 36	0 95	0 41
32	142	1 26	0 099	1 79	0 62	1 17
33	36	0 45	0 058	0 16	0 07	0 09
34	7	0 74	0 055	0 05	0 08	0 00
35	141	0 41	0 051	0 58	0 30	0 28
36	105	0 48	0 041	0 50	0 16	0 34
37	159	1 61	0 048	2 56	1 63	0 93
38	145	1 28	0 237	1 85	0 55	1 30
Mean	96	1 62	0 102	1 68	0 63	1 06
SD	±49	±1 46	±0 062	±1 91	±0 52	±1 56

ed in table II, averaged 0 102±0 062 This ratio expresses the glomerular permeability of haemoglobin relative to creatinine or the glomerular clearance ratio The rate of tubular reabsorption of haemoglobin varied between 0 00 and 5 74 mg/min, with an average value of 1 06 mg/min In the dog *in vivo* Tm is about 3–4 mg/min [13] Values of glomerular clearance ratio in Hb in the dog have been reported as 0 03–0 05 [13] in comparison with our average value of 0 10±0 06

Excretion of haemoglobin in diminishing urine flow This was studied in 12 perfusion experiments The urine flow diminished from 5 71±3 86 (average ± SD) ml/min to 4 19±3 53 ml/min At the same time the free plasma haemoglobin level increased from 121±113 mg% to 221±182 mg% The other results are listed in table III

Although in rising urine flow the calculated reabsorptive rate did not change with increasing loads, we see that with diminishing urine flow there is a decrease in reabsorptive rate, and no more reabsorption in 8 to 12 kidneys can be demonstrated The mean $P_{free\ Hb}$ level is higher

Table III Values averages and standard deviations of $P_{free\ Hb}$ $C_{free\ Hb}$, $C_{free\ Hb}/C_{Cr}$ filtered load, $U_{free\ Hb}$ V and $T_{free\ Hb}$ in 12 isolated canine kidney perfusion systems with diminishing urine flow

Experiment No	Averages $P_{free\ Hb}$, mg%,	$C_{free\ Hb}$, ml/min	$C_{free\ Hb}/C_{Cr}$	Filtered load mg/min	$U_{free\ Hb}$ V, mg/min	$T_{free\ Hb}$, mg/min
17	201	0 62	0 022	1 24	1 77	0 00
19	95	1 67	0 100	1 59	1 38	0 21
20	70	1 60	0 131	1 12	1 33	0 00
21	38	0 70	0 092	0 27	0.29	0 00
24	92	2 77	0 379	1 55	2 26	0 29
27	176	1 05	0 045	1 84	2.32	0 00
29	451	0 69	0 043	3 11	3 65	0 00
33	75	0 31	0 035	0 24	0 28	0 00
34	44	0 83	0 066	0 36	0 37	0 00
35	258	0 56	0 056	1 44	0 85	0.54
63	105	0 20	0 021	0 21	0 10	0 11
73	412	0 53	0 016	2.18	5 64	0 00
Mean	168	0 96	0 084	1 35	1 68	0 10
SD	±139	±0 72	±0 099	±0 96	±1 63	±0 18

than in table II, because the diminishing urine flow always followed the rising urine flow at the end of the perfusion That also resulted in an average lower $C_{free\ Hb}$, a lower filtered load and a higher urinary excretion of haemoglobin

The relationship between the excretory rate and the plasma level of free haemoglobin LAMBERT *et al* [13] have demonstrated that the graphic relationship between urinary excretion and plasma levels of free haemoglobin was linear The urinary excretion of free haemoglobin *increased progressively and proportionately* as the plasma level of free haemoglobin increased In figure 1, 10 representative experiments of this linearity in isolated kidney perfusion are plotted graphically We found this linearity in 18 of 20 experiments The slope of the curves was obtained by calculating the linear regression equation between the determined values of $U_{free\ Hb}$ V/C_{Cr} and $P_{free\ bH}$ The intercept of the horizontal axis was obtained by extrapolation of the regression line for $X \equiv 0$ in $Y = a + bX$ The intercept of the horizontal axis represents the theoreti-

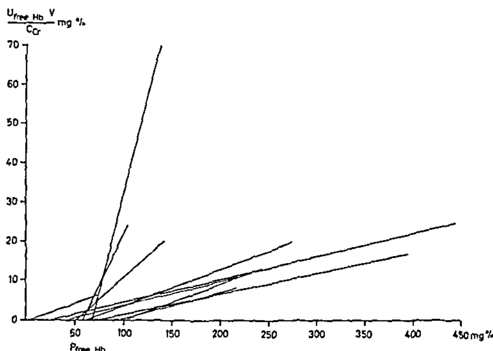


Fig 1 The relationship between the excretory rate and the concentration of free haemoglobin in plasma (10 representative experiments)

cal plasma level of free haemoglobin above which haemoglobinuria occurs This value is represented by 'a' in the linear regression equation Table IV gives the linear regression equations of the relationship between *the excretory rate and plasma concentration of free haemoglobin* in 18 isolated kidney perfusions

These values are considered as theoretical estimates only since the relationship between $U_{free\ Hb}$ V/C_{Cr} and $P_{free\ Hb}$ at low values (<1 mg/min) was undetermined Within these limits and those imposed by the accuracy of estimating the relationship of $U_{free\ Hb}$ $V/P_{free\ Hb}$ the values obtained for $P_{free\ Hb}$ (the theoretical plasma level above which haemoglobinuria occurred) varied in individual studies from 5 7 to 119 2 mg% In dogs *in vivo* accumulation of free haemoglobin in plasma resulted in haemoglobinuria only when levels of approximately 70 mg% or more were present [1] Others [18] found haemoglobinuria with free plasma haemoglobin values between 57 and 83 mg% Our values correspond very well with those found in dogs *in vivo*

Table IV The linear regression equations of the relationship between the excretory rate and plasma concentration of free haemoglobin in 18 isolated kidney perfusions

Kidney No	Linear regression equation Y = a + bX	Duration of haemoglobinuria min
17	29 9 + 20 0 X	150
19	50 5 + 4 6 X	240
20	36 4 + 2 6 X	150
21	8 4 + 2 2 X	90
23	119 2 + 0 5 X	120
24	67 2 + 1 0 X	210
25	58 3 + 1 9 X	90
26	93 0 + 3 7 X	270
27	62 6 + 10 5 X	300
28	40 9 + 16 0 X	120
29	104 3 + 10 5 X	210
31	29 8 + 16 5 X	270
32	95 0 + 10 0 X	120
33	57 5 + 4 6 X	90
34	5 7 + 11 3 X	60
35	117 6 + 13 4 X	210
36	97.5 + 14 8 X	210
37	78 7 + 18 5 X	360

Discussion

The examination of our data reveals that the isolated kidney handles haemoglobin in the same manner as the kidney *in vivo* Protein-bound haemoglobin is not excreted in the urine The rate at which free haemoglobin is excreted depends upon 3 variables (1) the plasma level of free haemoglobin, which varied from 0 to more than 600 mg%, (2) the rate of clearance of free haemoglobin from plasma by the glomerulus, which varied from 1 62 ± 1 46 ml/min in rising urine flow to 0 96 ± 0 72 ml/min in diminishing urine flow, and (3) the rate of tubular reabsorption, which varied from 0 00 to 5 74 mg/min in rising urine flow The filtered load of haemoglobin is lower in experiments with diminishing urine flow (1 35 ± 0 96 mg/min) than in experiments with rising urine flow (*1 68 ± 1 91 mg/min*), because the relative formation of haemoglobin subunits is enhanced by decreasing concentration [4], which is lower in experiments with rising urine flow

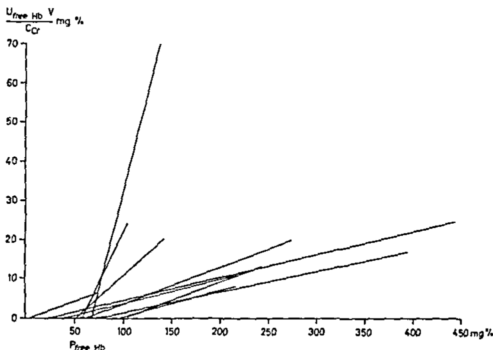


Fig 1 The relationship between the excretory rate and the concentration of free haemoglobin in plasma (10 representative experiments)

cal plasma level of free haemoglobin above which haemoglobinuria occurs This value is represented by 'a' in the linear regression equation Table IV gives the linear regression equations of the relationship between the excretory rate and plasma concentration of free haemoglobin in 18 isolated kidney perfusions

These values are considered as theoretical estimates only since the *relationship between* $U_{free\ Hb}$ V/C_{Cr} *and* $P_{free\ Hb}$ *at low values* (<1 mg/min) was undetermined Within these limits and those imposed by the accuracy of estimating the relationship of $U_{free\ Hb}$ $V/P_{free\ Hb}$ the values obtained for $P_{free\ Hb}$ (the theoretical plasma level above which haemoglobinuria occurred) varied in individual studies from 5 7 to 119 2 mg% In dogs *in vivo* accumulation of free haemoglobin in plasma *resulted in haemoglobinuria only when levels of approximately 70 mg%* or more were present [1] Others [18] found haemoglobinuria with free plasma haemoglobin values between 57 and 83 mg% Our values correspond very well with those found in dogs *in vivo*

Table IV The linear regression equations of the relationship between the excretory rate and plasma concentration of free haemoglobin in 18 isolated kidney perfusions

Kidney No	Linear regression equation Y=a+bX	Duration of haemoglobinuria, min
17	29 9+20 0 X	150
19	50 5+ 4 6 X	240
20	36 4+ 2 6 X	150
21	8 4+ 2 2 X	90
23	119 2+ 0 5 X	120
24	67 2+ 1 0 X	210
25	58 3+ 1 9 X	90
26	93 0+ 3 7 X	270
27	62 6+10 5 X	300
28	40 9+16 0 X	120
29	104 3+10 5 X	210
31	29 8+16 5 X	270
32	95 0+10 0 X	120
33	57 5+ 4 6 X	90
34	5 7+11 3 X	60
35	117 6+13 4 X	210
36	97 5+14 8 X	210
37	78 7+18 5 X	360

Discussion

The examination of our data reveals that the isolated kidney handles haemoglobin in the same manner as the kidney *in vivo* Protein bound haemoglobin is not excreted in the urine The rate at which free haemoglobin is excreted depends upon 3 variables (1) the plasma level of free haemoglobin, which varied from 0 to more than 600 mg%, (2) the rate of clearance of free haemoglobin from plasma by the glomerulus, which varied from 1 62±1 46 ml/min in rising urine flow to 0 96±0 72 ml/min in diminishing urine flow, and (3) the rate of tubular reabsorption, which varied from 0 00 to 5 74 mg/min in rising urine flow The filtered load of haemoglobin is lower in experiments with diminishing urine flow (1 35±0 96 mg/min) than in experiments with rising urine flow (1 68±1 91 mg/min), because the relative formation of haemoglobin subunits is enhanced by decreasing concentration [4], which is lower in experiments with rising urine flow

Free haemoglobin did not appear in the urine until after plasma hae moglobin level had exceeded the binding capacity of haptoglobin As measured by factor a in the linear regression equation in table IV, the theoretical plasma level of free haemoglobin above which haemoglobi nuria occurred averaged 64 0±35 2 mg%

In the present study renal tubular reabsorption of unbound haemo globin averaged 1 06±1 56 mg/min in 13 isolated individual kidneys We must multiply these data by 2 (for 2 kidneys = 2 12±3 12 mg/min) to compare our results with those of Monke and Yuile [15] Lambert *et al* [13] and Vandervelken *et al* [19] which were in the dog *in vivo* respectively 2 09±0 23 4 73±2 49 and 3 04±1 83 mg/min We found the same average value as Monke and Yuile [15] but with a greater stand ard deviation

The estimation of tubular reabsorption of haemoglobin is based on the arithmetic difference between 2 variables filtered load and urinary excretion Because of the errors inherent in the measurement of these 2 variables estimation of the magnitude of tubular reabsorption shows considerable variability with a range of 0 00–5 74 mg/min It should be noted that in every kidney with rising urine flow except one there was some physiologic evidence of reabsorption of haemoglobin The figures of the linear regression equation are therefore more suggestive of tubu lar reabsorptive activity than are the calculated reabsorptive rates on the assumption that it was necessary to elevate the plasma concentration of free haemoglobin to these levels before tubular reabsorptive activity was saturated and haemoglobinuria occurred

It is difficult to explain why we could not calculate tubular reabsorp tion of haemoglobin in 8 of 12 kidneys with decreasing urine flow One explanation was that there was really no reabsorption because the dim inishing urine flow usually arrived at the end of the perfusion experi ment when the functional capacity of the isolated perfused dog kidney was very low [9] It is also more likely that it is an error in calculation in part related to different changes in $C_{free\ H}$ glomerular filtration rate and filtered load Others [4] observed an inverse relationship between renal uptake and the concentration of haemoglobin which was higher in the experiment with diminishing urine flow

We can agree with Rabiner *et al* [17] that it was possible to infuse stroma free haemoglobin solution without any acute deleterious effects on renal function because we found no influence in blood or plasma flow glomerular filtration rate and extraction ratio of *p* aminohippurate

[9] However, in individual experiments, in which no stroma-free haemoglobin solution was infused, the progressive haemolysis of the perfusion blood resulted in the production of a hypercoagulable state, because haemolysed erythrocytes can initiate blood coagulation [18], which explained the fall in blood flow and creatinine clearance in experiments with diminishing urine flow This was found by GOLDBERG [11] following administration of homologous haemolysed blood cells to dogs

Since we have a limited quantity of haemoglobin (the blood volume in the perfusion apparatus is always ±450 ml), we see that although the rate of haemolysis in general increases progressively with increasing duration of perfusion, the renal excretion of free haemoglobin was neither a useful nor efficient mechanism for elimination

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Acta haemat 47 109–117 (1972)

Endocrine Studies in a Case of Congenital (Erythroid) Hypoplastic Anaemia

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Abstract Congenital (erythroid) hypoplastic anaemia was diagnosed in a newborn male infant whose progress was followed until death at the age of 18 years He received a total of 456 units of blood and developed obvious transfusion haemosiderosis He showed total arrest of growth from the age of 9 years and remained sexually infantile These features are common accompaniments of this haematological disorder In addition, our patient became severely hypothyroid Endocrine studies provided limited evidence for an anterior pituitary lesion and at *post mortem*, the pituitary gland in common with all the internal organs, was found to contain haemosiderin deposits The suggestion is made that endocrine features in this condition are secondary to iron overload rather than part of the fundamental disorder A therapeutic trial of human growth hormone may prove of value in such patients

Key Words
Congenital anaemia
Growth hormone
Haemochromatosis
Hypothyroidism
Pituitary hypofunction
Transfusion haemosiderosis

Stunting of growth and failure of sexual maturation are commonly associated with a rare haematological disorder of childhood, congenital (erythroid) hypoplastic anaemia [1] This paper records the first detailed endocrine investigations in a classical case

Case Report

Our patient was the second of 4 children of healthy unrelated parents and there was no family history of endocrine or haematological disease A severe normochromic non haemolytic anaemia was present at birth Hb 7 g/100 ml) Numerous bone marrow biopsies revealed total or near total absence of erythroid cell precursors. A wide range of haematinics was exhibited (table I) without demon

Table I Haematinic therapy

Date started	Drug	Dose	Duration
November 1950	vitamin B_{12}	20 μg/day	1 month
December 1950	folic acid	20 mg/day	1 month
January 1951	testosterone propionate	1 25 mg i m twice weekly	2 weeks
	later	*2 5 mg i m twice weekly*	*3 weeks*
February 1951	testosterone propionate	2 5 mg i m once weekly	4 weeks
February 1951	vitamin B_{12}	20 μg/day	4 years
February 1951	pyridoxine	100 mg/day	1 month
March 1951	McKay's mixture (iron, thyroid extract, ascorbic acid and folate)		2 months
January 1952	cortisone	15 mg t i d	3 weeks
January 1952	cortisone	50 mg/day	3 weeks
April 1953	placental plasma	140 ml i v	once only
	placental plasma	60 ml i v	once only
November 1953	cortisone	100 mg/day	4 weeks
December 1954	ACTH	80 units/day	15 days
March 1957	prednisolone	10 mg b d	2 months
May 1957	prednisolone	5 mg b d	1 month
April 1962	prednisolone	5 mg t i d	6 months
December 1962	fluoxymesterone	1 mg b d	6 weeks
	triamcinolone	4 mg b d	
January 1963	triamcinolone	4 mg b d	9 months
	methyltestosterone	20 mg b d (sublingual)	
September 1964	prednisolone	5 mg b d	1 month
October 1964	prednisolone	2.5 mg b d , until death 4 ½ years late	

strable effect and regular transfusions were required from the age of 3 weeks During the 18 years of his life he received 456 units of blood, mainly as packed cells.

In infancy he proved unduly susceptible to bacterial infection This was associated with a poor leukocyte response and in later years he developed a marked neutropenia (2,000 white cells/mm³, 55–70% lymphocytes) In contrast, he seemed very resistant to viral infection and never suffered from any of the common exanthemata Presumably this was attributable to his regular infusion of γ globulin in transfused blood Thrombocytopenia developed from the age of 8 years and during the last 5 years of his life the ensuing haemorrhagic tendency was controlled only by continuous steroid therapy Splenectomy was offered but the patient s family refused permission for surgery

Bronzing of the skin was noted from the age of 2 years and progressive hepatosplenomegaly developed from the age of 9 years In 1963 (age 13 years) he be

came deeply jaundiced and liver function tests were markedly disturbed It could not be established whether this was due to hepatitis or whether liver damage had been caused by the methyltestosterone which he had received over the preceding 9 months His jaundice subsided over a period of weeks when this drug was withdrawn A glucose tolerance test, undertaken while jaundice was still present, gave a frankly diabetic result but glycosuria disappeared as liver function returned to normal In 1966 a blood glucose level of 96 mg% was recorded 2 h after a meal and in 1968 the fasting level was less than 80 mg% There was, therefore, no clinical evidence of pancreatic islet cell damage

Involvement of the endocrine system was first suspected at 6 years when his growth rate began to decline Growth ceased altogether at 9 years when his height was only 120 cm. In 1963 (age 13 years) lethargy and deteriorating school performance suggested the onset of hypothyroidism. The thyroid gland was not palpable Serum protein bound iodine concentration was less than 1 μg/100 ml on 2 consecutive samples and the serum cholesterol was greater than 500 mg/100 ml Thyroxine was introduced and the dose slowly increased to 0 1 mg daily At this level he was clinical euthyroid although the PBI was only 3 4 μg/100 ml and serum cholesterol 290 mg/100 ml Increasing the thyroxine to 0 15 mg daily produced anxiety and sleep disturbance He, therefore, continued to take 0 1 mg daily for the rest of his life This therapy produced no change in height

Development of the gonads and secondary sexual characteristics remained infantile and even after prolonged administration of androgens in substantial doses he showed no evidence of virilisation At the age of 18 the upper/lower segment ratio was 1 15 1 and the bone age (left wrist) was 12 years Chromosome analysis of peripheral blood and of skin (Dr D G HARNDEN, Miss J M TOUGH) revealed no abnormality

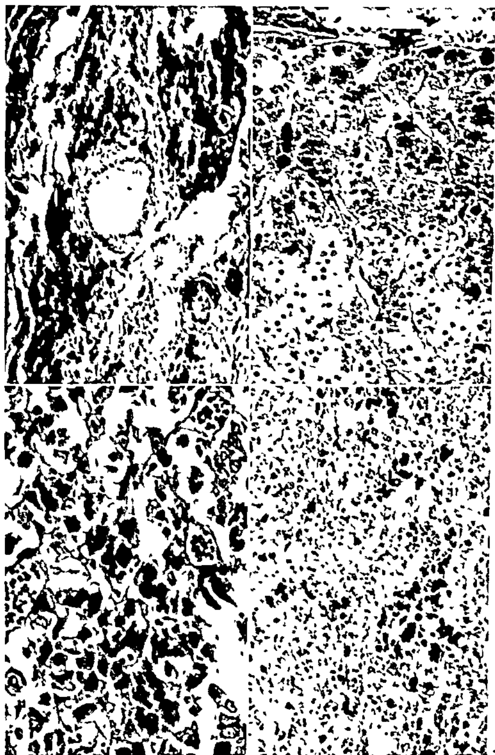
In February 1969 the patient suffered a massive gastro-intestinal haemorrhage and a severe chest infection He recovered slowly from both but shortly after discharge home he had a grand mal convulsion, in the course of which he sustained an intertrochanteric fracture of the right femur He died a few hours after re-admission to hospital

Pathological Findings

Necropsy was performed 31 h after death. All the internal organs with the exception of the central nervous system showed heavy brown pigmentation. The testes were small (1.5 cm max. diameter) and incompletely descended, occupying positions about halfway down the lateral walls of the scrotum The pancreas was hard and densely pigmented Only a small remnant of thyroid tissue was found. The adrenals showed marked cortical atrophy The pituitary was of normal dimensions but was pigmented

Microscopically the pancreas showed heavy iron deposition both in the pancreatic cells and in the intralobular fibrous tissue The thyroid was largely replaced by fibrous tissue, much of it heavily impregnated by iron and only a little colloid could be seen (fig 1) The adrenal showed thinning of the cortex and iron deposition, most marked in the outer layers (fig 2)

In the pituitary, heavy haemosiderin deposits were present, most of the cells contained it and in some it was so dense as to obscure the staining reaction (fig 3,


1
2
3
4

4) These heavily affected cells tended to occur in groups Generally the basophils seemed to contain most iron while the acidophils and chromophobes were less severely damaged

In the spleen heavy iron deposits were associated with an increase in fibrous tissue The liver showed a well developed portal cirrhosis Fat emboli were found in the lungs and brain

Endocrine Investigations

The combination of impaired growth sexual infantilism and non go trous hypothyroidism suggested a les on of the anterior pituitary and the investigations detailed in table II were therefore undertaken The results were interpreted as follows

Hypothalamic/pituitary/adrenal axis Rises in urine 24-hour 17 hydroxy corticosteroids following ACTH and metopirone were similar implying that the pituitary retained some capacity for ACTH production The actual level of response was low but was undoubtedly influenced by the patient s low body weight (29 kg) and by the fact that he had re eived continuous steroid therapy for 3 years until 1 week before the test.

Thyroid funct on The exceed ngly poor uptake of [131]I appeared resistant to TSH administration This would imply that the primary defect lay in the thyroid gland itself However it is well recognised that in long standing cases this test may fail to detect a primary lesion of the pituitary with secondary involution of the thyroid [6]

Pituitary hormone assays The urinary gonadotrophin output was well below the expected level for age and sex [Dr E. T Bell personal commun] implying pituitary insufficiency This appeared to be confirmed by the absence of measurable growth hormone in a plasma sample taken 3.5 h after an oral glucose load [7] There was, however an apparently adequate growth hormone response to insulin hypoglycaemia

Discussion

The association of dwarfism and sexual infantilism with congenital (erythroid) hypoplastic anaemia has been taken as evidence that the underlying (presumably biochemical) lesion is a multisystem one, not con-

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Fig 1 Thyroid tissue Colloid scanty relative to interacinar fibrous tissue which is heavily impregnated with haemosiderin H E, × 120

Fig 2 Adrenal cortex Haemosiderin present particularly *in the outer layers* of the cortex H E, 200

Fig 3 Pituitary gland Haemosiderin depos tion obscures the *staining reaction* in many of the cells H E, 300

Fig 4 P tuitary gland Although dense staining is *present in only* a proportion of cells iron deposition is seen to be *widespread* Prussian blue reaction × 120

Table II Endocrine studies

August 1965

ACTH and metopirone test [2 3] prednisolone withdrawn 1 week before test, ACTH 40 U i m on day 2 Metopirone 420 mg 4-hourly orally for 6 doses on day 6

	Day						
	1	*2*	*3*	*4*	*5*	*7*	*8*
Urine 24 h OH-corticosteroids mg	1 33	2 05	5 52	3 6	1 57	3 7	4 1
Urine 24 h ketosteroids, mg	1 38	1 74	2 37	1 36	0 75	1 37	0 86

August 1965

Radioiodine tests (thyroxine withdrawn 1 week before tests) ^{131}I 4-hour uptake 3 2%, *24-hour uptake 1 9%, 4 hour uptake 1 day after 2 5 U TSH i m 1 2 9%*

September 1965

TSH 2 5 U i m I on days 1 and 4 5 U i m I on day 3 ^{131}I 4 hour uptake on day 5 5%

March 1967

Pituitary hormones assays 48 hour urine human pituitary gonadotropins (total) in urine <0 29 mg/24 h (using 2nd international preparation of HMG) (Dr E T BELL) Mouse uterus assay [4]

February 1967

Plasma growth hormone 3 5 h after 50 g glucose *per os* <2 ng/ml (Dr W M HUNTER) Radioimmuno assay [5]

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Insulin 2 U given i v in fasting state

Time min	Blood glucose, mg %	Plasma growth hormone, ng/ml (Dr W M HUNTER)
0	79	1 3
30	39	6 7
60	48	7 6
90	57	12

fined to the red cell precursors of the bone marrow [8] In the present case, absence of virilisation in response to androgens may be most easily explained by this interpretation but until the fundamental chemistry of the condition is better understood this hypothesis cannot be proved

Alternatively, the endocrine involvement may be a secondary consequence of chronic anaemia or of its treatment The metabolism of hor-

mones is, for example, disturbed in advanced liver disease and our patient had long-standing hepatomegaly, one episode of frank jaundice and necropsy evidence of cirrhosis On the other hand, biochemical tests of his liver function, including plasma protein analysis gave normal results even in his last year of life and he developed no clinical stigmata of liver failure (palmar erythema, spider naevi or gynaecomastia) It, therefore, seems highly improbable that liver damage could have been a major factor in the inhibition of growth and sexual development over the preceding 10 years

Steroid therapy in children is commonly associated with stunting of growth by interfering with the peripheral action of growth hormone [9, 10] or by inhibition of its release [11, 12] In the present case steroids were administered almost continuously from the age of 11 5 years and may have contributed to the failure of growth from that stage However, deviation from the standard height curve was obvious by the age of 7 and growth appeared to be arrested completely by the age of 9 This cannot reasonably be attributed to the 3-month course of prednisolone administered during his 7th year No other steroids were given during the succeeding 5 years

The anatomical evidence of iron deposition in the pituitary and other glands and the results listed in table II suggest that in our patient dwarfism, sexual infantilism and hypothyroidism may have been a consequence of wide-spread involvement of the endocrine organs in transfusion haemosiderosis A similar conclusion was reached by ZAINO *et al* [13] who studied 7 children and young adults with thalassaemia major though in none of their patients was the endocrine disturbance so pronounced as in the present case Pituitary damage has been noted in adults with haemochromatosis [14] and the combination of diabetes mellitus and hypopituitarism with thalassaemia intermedia in a single reported case has been attributed to iron overload [15] In the relatively small number of patients who require regular blood transfusions from an early age, the risk of secondary endocrine damage should therefore, be borne in mind

Spontaneous or steroid induced remission in congenital (erythroid) hypoplastic anaemia even when it occurs after the expected age of puberty can be followed by an increase in growth rate and normal sexual maturation [1] This implies that the endocrine lesions are incomplete and potentially reversible, emphasising the need for an effective means of removing excess iron from the body Formal trials of several chelat

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ing agents alone and in various combinations were undertaken in our patient These included EDTA with ascorbic acid, DTPA (diethylamine, triamine, penta acetate, Geigy) and desferrioxamine This last agent, when added to transfused blood (1 g per unit, 3 units per transfusion) cleared up to 20 mg of iron in the urine over the next 48 h The other regimes were less effective and continuous therapy with any combination resulted in an average iron excretion of less than 2 mg/24 h Thus we were unable to make any significant contribution towards reducing the estimated iron load of 200 mg per unit of blood Since all the chelating agents were associated with side effects (painful injection sites, anorexia or skin eruptions) such measures were eventually discontinued

ZAINO *et al* [13] found no evidence of impaired somatotropin production in their patients and a trial of exogenous human growth hormone (HGH) in one failed to promote growth They concluded that the peripheral action of the hormone is inhibited in transfusion haemosiderosis Our patient had a very low fasting level of growth hormone which failed to rise after a glucose meal, strongly suggesting inadequate release of the material from the pituitary [7] In response to a potent hypoglycaemic stimulus, however, there was a measurable rise in circulating growth hormone levels In view of the scarcity of HGH it was felt that this last result precluded a trial of the hormone in our patient As the material becomes more readily available, a trial of its effect in such a case would seem to be amply justified not only from the endocrine point of view but also because there is evidence from animal work that pituitary hormones may have an influence on haematopoiesis [16, 17]

Acknowledgements The authors are glad to acknowledge the important contributions made to the management investigation and documentation of this patient throughout his clinical course by numerous paediatricians haematologists biochemists and research endocrinologists Particular mention must be made of Prof J O FORFAR Dr J SYME Dr E B FRENCH Dr S H DAVIES Dr N C ALLAN Dr S L TOMPSETT Dr D B HORN Dr T E BELL and Dr W M HUNTER Prof J A STRONG offered valuable advice and encouragement in the preparation of this paper Mr NORMAN DAVIDSON prepared the photographic plates and the secretarial work was kindly undertaken by Miss L RITCHIE

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Acta haemat 47 118–126 (1972)

Thalassaemia Major with Complete Suppression of HbA Production in a Ghanaian Girl[1 2]

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Key Words
Cooley anaemia
Haemoglobin electrophoresis
Haemoglobin F
Thalassaemia in Africa
Thalassaemia gene

Abstract A 7 year-old Ghanaian girl showed severe hypochromic anaemia hepatosplenomegaly widening of the diploic space and a hair on end appearance on a skull X ray Hb electrophoresis revealed only one thick band which was HbF no HbA was present Both the mother and her brother are β thalassaemia traits it was impossible to investigate the father Very few communications are available on thalassaemia major among Negroes The view is expressed that there is a heterogeneity of the thalassaemia gene in Africans and all the cases published previously have HbA in addition to HbF The present case belong to another group where the β chain is suppressed completely It is assumed that in Africa children with thalassaemia major die in early childhood without proper diagnosis and only those survive who have a milder course however those may also die before they reach the end of the first decade Further case reports and clinical and laboratory studies are necessary to obtain more information on thalassaemia major in Africans

The presence of β thalassaemia gene in Africa south of the Sahara has been established The frequency of genes of the β thalassaemia complex was estimated as 0 02–0 03 in Liberia Ivory Coast and Upper Volta [1] and 0 004 in Ibadan Western Nigeria [2] The frequency of thalassaemia trait was found to be 2% in the Congo [3] less than 1% among Karamojas in Uganda [4] and 4–5% in Northern Ghana [5] The incidence of β thalassaemia trait among American Negroes varies between 0 8 and 2% [6] A fair number of sickle cell thalassaemia and HbC thalassaemia cases have been published on both sides of the Atlan

1 Supported by a research grant of the World Health Organization

2 Authors recently got access to the paper of CABANNES *et al* (Revue de l Association des Medecins de Langue Française *4 180–188 (1968)* in which these authors present 4 cases of thalassaemia found in Ivory Coast

tic Ocean, but very few case reports are available on thalassaemia major among Africans This is an enigma and ESAN [2] speculated that this hiatus which also exists in Nigeria (where no patient with thalassaemia major has been found so far) is either due to failure of recognition or that the clinical presentation differs from that which is known as Cooley's anaemia in Caucasians

Recently we have seen a patient with thalassaemia major and because of the scarcity of data available on this disease in Africans and because of some unusual features we are presenting the case

Materials and Methods

Routine haematological techniques were used Agar gel electrophoresis was performed with citric acid-citrate buffer at pH 6 1 The quantification of HbA_2 was performed according to the method of MARENGO-ROWE [7] and elution technique of foetal haemoglobin according to KLEIHAUER *et al* [8]

Case Report

D A., a Ghanaian girl 7 years of age was admitted to the Department of Pediatrics Korle Bu Teaching Hospital Accra, with fever and severe abdominal pains in November 1970 There was no previous history of hospital admission, blood transfus on or serious disease On examination the patient s physical development was found to be retarded and the mucous membranes showed extreme

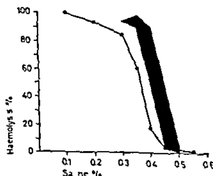


Fig 1 Osmot c fragility curve decreased fragility

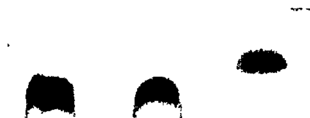


Fig 2. Agar gel electrophoresis, pH 6.1. Left: control HbAS, middle: mother's blood, HbAA; right: patient's blood, HbF. Note the absence of HbA.



Fig 3 Skull X-ray, hair on end appearence



Fig 4 Tibia with thinned cortex and increased trabeculation.

paleness Frontal and parietal bossing was visible and both the liver and the spleen were considerably enlarged the lower end of the liver was palpable 10 cm and that of the spleen 15 cm below the costal margin Hb 4 8 g%, PCV 18%, WBC 6,000 Sickling test negative Retics 3.2% Blood film the red cells show marked hypochromia, anisocytosis, leptocytosis and fragmentation with a fair number of polychromatophil cells and a few target cells 13 nucleated red cells were found per 100 WBC. G-6PD was normal Serum iron 50 μg% and TIBC 206 μg%. There [illegible] 3 26 mg%

The patient was admitted and a pint of blood was given as well as antibiotics. After a few days the temperature subsided and the general condition improved, she was discharged on 5 mg folic acid daily and antimalarials. There was a regular follow up in the Out Patient Department and when the child came on 4th February, 1971 the Hb had fallen to 4.2 g% and blood transfusion was given again. This was followed by a rise of the Hb to 7 6 g%. On 3rd March the Hb was 6.3 g%, on 16th April 6 0, and on 3rd May 5.5 g%. A third transfusion was given

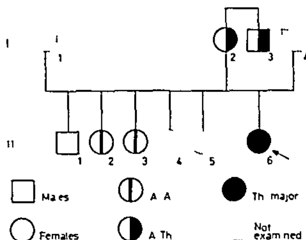


Fig 5 Family tree

on that day On 14th June the Hb was found to be 5 0 g% and she was retransfused

The Hb electrophoresis on a blood sample taken before transfusion on agar gel electrophoresis pH 6 1 showed a single band which could be identified as HbF (fig 2) No trace of HbA was seen HbA_2 was 2 6% and the one minute alkali resistant haemoglobin test gave the fetal Hb percentage as 88 There was no elution of haemoglobin from the red cells using the KLEIHAUER BETKE test On a skull X ray the diploic space in the skull was greatly widened and a hair on end appearance was present (fig 3) The cortex of the long bones was thinned and increased trabeculation could be observed (fig 4)

Family study The mother of the patient is a Ghanaian woman without any complaint or symptoms of disease She has 6 children of which the propositus was the youngest the other 5 children have a different father Despite every effort it was impossible to trace the father of the propositus The mother and the mother s brother have increased HbA_2 with no elevated HbF and the peripheral film showed slight microcytosis hypochromia and occasional target cells None of the half brothers or sisters has increased HbA_2 or F or morphological changes in peripheral blood Figure 5 shows the family tree and table I the important laboratory results

Discussion

The diagnosis of thalassaemia major in our case was based on the very severe hypochromic anaemia haemolysis hepatosplenomegaly, bone changes and the presence of HbF as the only major haemoglobin The family study has shown that the mother and her brother have the

thalassaemia trait Unfortunately it was impossible to examine the father The KLEIHAUER BETKE test revealed that the red cells of the propositus contained only HbF, there being no elution, this was so because there was no other Hb, apart from HbA_2 in the red cells A similar finding, i e non-elution of haemoglobin with acidic buffer from the red cells has also been seen by one of us [9] in an adult patient who was homozygous for the gene of hereditary persistence of foetal haemoglobin (HPFH) In the present case, however, HPFH could be ruled out because there was no evenly distributed HbF in the mother's red cells, so the propositus could not inherit persistent high F gene from both of her parents.

Publications on thalassaemia major among Negroes are surprisingly few In Africa 2 cases were reported from Liberia by OLESEN *et al* [13] and one case from Ghana [10] A case was mentioned from the Congo [3] and this was quoted as evidence of the presence of the thalassaemia gene in Central Africa [11], nevertheless we could not trace any further report on this case and later it was stated that in the Congo only thalassaemia minor and sickle cell thalassaemia have been observed [12] The two Liberian cases were published in 1959 but the clinical and laboratory data are scanty One patient was a 5-year-old child with severe hypochromic and microcytic anaemia who underwent splenectomy Hb was 35% and on electrophoresis HbA and F were present, the percentage of the latter was 35 No clinical data are available on this case The second child had a moderate anaemia (Hb 7 g%), electrophoresis was not done and the diagnosis was made after death of the child However, the family study suggested that this second child might have been a heterozygote for hereditary persistence of fetal haemoglobin

The patient from Ghana published by BOI-DOKU and OFORI ATTA in 1967 [10] was a 3-year-old boy with severe microcytic hypochromic anaemia, Hb 5 g%, PCV 23% On electrophoresis both HbA and F were present, the alkali resistant Hb test revealed 55% fetal haemoglobin A_2 was 5%, the mother had 4 5% and the father 6 7% HbA_2

There are a few communications on thalassaemia major among Negroes in the New World WENT and MACIVER [14] in the West Indies described 6 cases in 4 families Two of these patients were adults aged 48 and 30 years of age, they had mild anaemia, the Hb being 7 3 and 9 0 g%, respectively The age of the 4 children varied between 14 months and 2 ½ years and 3 individuals had more severe anaemia, and Hb was between 4 7 and 5 g% In all 6 cases HbA and F were present

Table I

o of mily ee	Age years	Position in the family	Hb g/100 ml	HbF %	HbA_2 %	Blood film	Hb elution test	Genotype
3	42	uncle	14 2	0 6	4 5	moderate hypochromia target cells	uneven distribution	β thalass. minor
2	39	mother	12 6	1 7	5 5	moderate hypochromia target cells	uneven distribution	β-thalass. minor
6	7	propositus	4 5	88[1]	2 6	severe hypochromia microcytes fragmentation target cells nucleated red cells	no elution	β-thalass. major

[1] At very high concentration the measurement of HbF by alkali resistance gives erroneously low re

HELLER *et al* [15] observed 2 sisters, both adults of 39 and 37 years of age with moderate anaemia the HbF was 62 and 34% The 2 latter patients and the 2 adults in WENT and MCIVER's paper correspond to the clinical form of thalassaemia intermedia which was described in the United States [16] The 4 children from the West Indies show the classical picture of thalassaemia major

In connection with our patients we would like to comment on 2 points Thalassaemia major is usually diagnosed in the first 18 months of life [17] The child was brought to us when she was 7 years old and according to her mother she had not been ill until that time and transfusion had not been given to her This is strange because since we have seen her she had needed transfusion every second month or so to maintain an Hb level around 7 g% It is not clear whether a triggering mechanism exists which aggravated the disease The second point is that when we investigated the blood first before transfusion there was only HbF as major Hb present, no trace of HbA could be demonstrated Later, after transfusions there was also HbA visible but this came from the transfused blood

It seems that there is a heterogeneity of the thalassaemia gene in Negroes Apart from thalassaemia intermedia there are at least 2 forms one is characterized by the presence of HbA besides HbF and it appears that the gene which produces the β-chain is not suppressed completely The cases found among Negroes previously belong to this form There is another form however, in which the β-chain producing gene is completely suppressed, i e there is no HbA Our case is the only one up to now which represents this group As far as the clinical course is concerned, both show severe anaemia hepatosplenomegaly and hyperplasia of the bone marrow If further observations become available perhaps a better understanding can be obtained of the pathophysiology and effect of the 2 forms of thalassaemia major

With a relatively high percentage of the thalassaemia gene in West Africa and other parts of the continent more cases of thalassaemia major may be expected The disease in Europe and the United States is most commonly diagnosed by the pediatrician in early childhood It has been suggested that environmental factors may modify the expression of the gene and thalassaemia intermedia is a result of such modification [18] This could explain why the clinical presentation of thalassaemia major is different among Negroes It is quite possible, however, that not infrequently children in Africa with obscure anaemia and various diagnosis in fact die as a result of thalassaemia major Only those cases survive who for some reason or other have a milder course but probably they also die before they reach the end of the first decade Further clinical and laboratory observations are needed to cast light upon the various factors which affect the prevalence course and prognosis of the disease in Africa

Acknowledgement Authors wish to express their gratitude to Dr Y Asirifi for giving permission to publish the case

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E. Gautier und L.-S. Prod'hom (Hrsg.): **Hämatologie im Kindesalter.** Pädiatrische Fortbildungskurse für die Praxis, Vol. 31. Hrsg. E. Rossi. Karger, Basel 1971. 125 pp., 54 fig., 35 tab.; US $ 8.–/sFr. 35.–

In diesem neuesten Band der Serie der Pädiatrischen Fortbildungskurse für die Praxis werden von namhaften Autoren die neuesten Fortschritte einiger wichtiger Teilgebiete der pädiatrischen Hämatologie besprochen. Unschätzbaren Wert besitzen die Beiträge über Physiologie und Pathologie der Erythrozyten, die beiden Arbeiten sind kurz, sehr weit umfassend und auf den allerneuesten Stand der Kenntnisse gebracht. Trotzdem sind sie gleichzeitig auch für den praktizierenden Arzt verständlich. Auf die Prophylaxe der Rhesuskrankheit und die praktische Durchführung der Therapie mit Anti-D-Globulin wird in einem weiteren Kapitel eingegangen, das für den Kliniker von grösstem praktischem Wert ist. Vollständig werden Differentialdiagnose und Therapie der Gerinnungsstörungen des Neugeborenen diskutiert. Allerdings wird die Hypothese eines Zusammenhanges zwischen «respiratory distress syndrome» und Verbrauchskoagulopathie nur von wenigen Neonatologen geteilt. Für den praktizierenden Arzt vorläufig nur von theoretischem Interesse ist die Zusammenfassung über neuere Erkenntnisse der Leukozytengruppen. Erst in letzter Zeit weiss man mehr über die Leukozytenfunktion und deren Störungen. Sowohl methodisch wie auch klinisch erhält man in einem weiteren Artikel in Kürze ein Maximum an wertvollen Informationen. Im letzten Beitrag wird die sich sehr rasch entwickelnde moderne Therapie der akuten Leukämien kursorisch besprochen und mit Recht ein optimistisches Zukunftsbild gezeichnet.

Zu erwähnen bleibt, dass sich fast jedem Kapitel eine wertvolle und nützliche Bibliographie anschliesst. Obschon das Büchlein nur ein enges Spektrum der Fortschritte in der Kinderhämatologie umfasst und trotz des relativ hohen Preises, ist es von grösstem Wert sowohl für den Allgemeinmediziner wie auch für den Spezialisten und verdient daher eine weite Leserschaft.

J. Sartorius, *Basel*

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The 11th International Cancer Congress organized under the auspices of the UICC will be centered in Florence October 20–26, 1974 President PIETRO BUCALOSSI, General Secretary UMBERTO VERONESI

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For information write to 11th International Cancer Congress Istituto Nazionale per lo Studio e la Cura dei Tumori Via G Venezian 1, *20133 Milano* (Italy)

Acta haemat 47 129–133 (1972)

Metabolic Aspects of Leukemic Lymphocyte Immune Reactivity[1]

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Abstract The leukemic lymphocyte has a limited ability to appropriately raise its metabolism under selected stimuli (phytohemagglutinin [PHA] and methylene blue [MB]), a correlative and interdependant relationship exists between glucose utilization and polynucleotide formation The results of these studies suggest that the lymphocyte in chronic lymphocytic leukemia reacts immunologically in accord with limitations imposed upon it by inherent cellular deficiencies These restrictions may prevent *in vivo* maximal immunogenic response and effective participation in host defense

Key Words
Lymphatic leukemia
Lymphocyte immune reactivity
Lymphocyte metabolism
Phytohemagglutinin

The present study experimentally manipulates glucose utilization by normal and leukemic lymphocytes in cell culture after contact with phytohemagglutinin (PHA) and methylene blue (MB), pharmacologic agents known to influence pathways of glucose carbon flow, and concurrently quantitates nucleotide biosynthesis under the same laboratory conditions The results provide evidence for a correlative relationship between these two aspects of lymphocyte growth and development

Methods

Lymphocyte sources Normal lymphocytes were obtained from the professional and nonprofessional hospital staffs who volunteered as healthy lymphocyte donors Leukemic lymphocytes were provided by 7 patients with chronic lymphocytic leukemia

[1] Supported by USPHS Research Grant CA-07000

Experimental techniques Peripheral blood lymphocytes were harvested and placed in culture with PHA and MB as previously described [1, 2] Incorporation of ^{14}C-thymidine (^{14}C-Tdr) was taken as a reflection of DNA synthesis [3] The hexose monophosphate shunt (HMP) and Embden Meyerhof (E M) pathways of carbohydrate metabolism were measured using ^{14}C glucose as substrate by quantitating the evolution of $^{14}CO_2$ and the generation of radioactive collective glycolytic intermediates [4, 5]

Results

PHA stimulation resulted in the characteristic and predictable increased uptake of ^{14}C-Tdr by normal lymphocytes (mean 17,656 cpm) Exposure to MB alone reduced ^{14}C-Tdr incorporation to 20–37% of that accomplished by untreated cells Addition of MB to lymphocytes incubated with PHA interfered with the latter agent's action and reduced DNA synthesis to 60% of that observed when cells were stimulated only with the mitogen

Normal lymphocytes in plain culture consumed a mean of 3 6 nmoles glucose via the HMP shunt and 118 6 nmoles through the E-M pathway Exposure of the cells to PHA, which is known to stimulate both the HMP and E-M pathways [6], increased glycolysis (mean 262 nmoles) but the quantity of $^{14}CO_2$ generated (mean 2 9 nmoles glucose) appeared to fall Addition of MB increased the evolution of $^{14}CO_2$ to almost twice the baseline value (mean 6 9 nmoles) and caused a marked decrease in phosphorylated intermediates (mean 27 3 nmoles) Concurrent exposure to PHA and MB resulted in the greatest yield of $^{14}CO_2$ (mean 17 1 nmoles) and a 30- to 50 percent drop in the amount of sugar traversing the E-M pathway when compared with untreated cells

Quantitative data derived from the experiments which measured leukemic lymphocyte biosynthesis and metabolism were appreciably lower than comparable values for analogous assays with normal cells, although qualitative similarities between both cell groups were present Thymidine incorporation by leukemic lymphocytes in plain culture resulted in a mean pellet radioactivity of 136 cpm After leukemic lymphocytes were incubated with PHA a mean count of 2,610 cpm was produced The response to MB by leukemic lymphocytes resembled that of the normal cells Finally, the mean of 1,021 cpm of incorporated ^{14}C-Tdr after leukemic lymphocyte exposure to combined PHA and MB is approximately 10% of that accomplished by normal lymphocytes under identical conditions

The glucose assays demonstrate that carbohydrate metabolism of the leukemic lymphocyte is considerably less active than the identical function performed by normal cells Leukemic lymphocytes in cultures without additives utilized a mean of 1.5 nmoles glucose through a consistently depressed pentose phosphate pathway A mean glucose utilization of 78 2 nmoles glucose via the E-M pathway was lower than the parallel value for normal cells Under the influence of PHA, glucose utilization through the HMP shunt appeared to rise slightly in some instances when compared with shunt activity of the unstimulated cell Lymphocyte glycolysis rose 1 5–6 times when stimulated with PHA although the mean of 154 5 nmoles sugar consumed did not equal normal, PHA-provoked glycolysis of normal lymphocytes Addition of MB increased shunt activity in the leukemic lymphocyte but the mean of 3 5 nmoles glucose consumed through this metabolic route was lower than normal comparable values Glucose utilization through the E M pathway by neoplastic lymphocytes was depressed after dye exposure and was identical to that of the normal lymphocyte Finally, when leukemic lymphocytes were exposed to combined MB and PHA they developed highest HMP activity and intermediate glucose utilization through the E-M pathway These levels never approached values obtained with normal lymphocytes

Discussion

This investigation points out that the leukemic lymphocyte has a limited ability to appropriately raise its metabolism under selected stimuli. This was clearly shown, first, by the absolute quantities of $^{14}CO_2$ generated by leukemic compared with normal lymphocytes when exposed to MB, a hydrogen acceptor which augments the pentose phosphate pathway by providing more rate-controlling NADP derived from NADPH [7] In addition, leukemic lymphocyte metabolic response to PHA alone also was reduced compared with normal cells Finally, the comparative reaction of the neoplastic and normal lymphocyte following simultaneous exposure to MB and PHA points out even more overtly differences between the capacities of the HMP shunt and E M pathway in these two cell types This statement is consistent with previously reported depressed concentrations of individual shunt and glycolytic enzymes when they are measured in lymphocyte lysates [8]

An additional major observation of this study is that lymphocyte glucose metabolism and DNA synthesis appear to be interdependent phenomena [9]. This inferential conclusion is supported by the semiquantitative, correlative relationship demonstrated between lymphocyte glucose utilization and DNA synthesis after stimulation with PHA and the diminution of ^{14}C-Tdr incorporation which occurred when MB distorted the directions of glucose carbon flow. In the latter circumstance inhibition of DNA formation by the dye in the presence of the mitogen was most probably due to the lack of NADPH required for polynucleotide reduction and to the limited amount of ATP generated by a reciprocally-depressed E-M pathway following metabolic diversion. The restricted synthesis of nucleotides after exposure to MB cannot be ascribed to impaired cell viability, *per se*, since both the normal and leukemic lymphocyte adequately excluded erythrosin B as observed repeatedly in the laboratory. Similarly, augmentation of the HMP shunt is hardly consistent with immediate cell death. Rather, this synthetic suppression is secondary to focal interruptions of necessary biochemical interactions.

The present data suggest, based on PHA as a prototype stimulus which could be replaced *in vivo* by an infective antigen, that the leukemic lymphocyte reacts immunologically in accord with limitations imposed upon it by inherent cellular deficiencies. It appears to be unable to respond maximally to metabolic and ultimately biosynthetic stresses which are necessary for effective participation in body immunity [10].

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Acta haemat 47 134–139 (1972)

Mise en évidence des antigènes du système ABO sur les lymphocytes humains à l'aide de l'électrophorèse en phase liquide[1]

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Abstract The effect of anti A and anti B antibodies on the mobility of A B and O lymphocytes has been studied by means of liquid phase electrophoresis A reduction in electrophoretic mobility was observed when the antibodies were in the presence of the supposed antigens of the same specificity Since ionic strength remained constant throughout the experiment, these results demonstrate indirectly the existence of ABO system antigens on human lymphocytes.

Key Words
Liquid phase electrophoresis
Leukocyte blood groups
Lymphocyte antigens

Les études sur la charge des leucocytes restent à ce jour peu nombreuses Différents domaines d étude ont cependant été envisagés [19] C'est ainsi que l'on a pu appliquer cette technique à l'étude théorique de la membrane [2, 13, 20, 21], à certains études immunologiques [4, 7] ou à la recherche de variations pathologiques éventuelles des éléments figurés de la lignée blanche [9, 10, 12] Nous mêmes avons étudié et précisé l influence du pH et des anticorps cytotoxiques sur la charge lymphocytaire [8, 18] Par contre, l influence des anticorps du système ABO sur la mobilité électrophorétique des lymphocytes n'a jamais, à notre connaissance, éte envisagée [17]

Le but de ce travail est de mettre en évidence l'action des anticorps anti A de B et anti-B de A sur les lymphocytes prélevés chez des individus de phénotypes érythrocytaires A, B ou O, afin de montrer, par l électrophorèse en phase liquide, que les antigènes considérés sont portés par cette variété de cellules sanguines Pour des raisons d approvisionnement en

[1] Ce travail a été réalisé avec l aide de la DRME (section biologie) contrat no 71.34 010 00 480 75 01

suspensions lymphocytaires, nous n'avons pas étudié à ce jour des lymphocytes prélevés sur des donneurs de groupe AB

Il est certain cependant que la presence des antigenes du système ABO sur les leucocytes n'est pas contestée [5] et que de nombreux travaux depuis 1955, à l'aide de méthodes expérimentales diverses, l ont prouve Parmi ceux-ci, rappellons ceux de Rlls [11], par la réaction d'agglutination, BERROCHE et coll [3] par le test d adsorption-elution, enfin ceux de GURNER et COOMBS [6] qui le montrent par une reaction d'agglutination mixte érythrocytaire D'autres auteurs, comme ANDERSON et WALFORD [1], ont également prouve l'existence des antigènes ABH sur les eléments blancs du sang, mais non sur les lymphocytes en particulier

Materiel et méthode

Séparation des lymphocytes Nous avons utilisé la technique rapide de HARRIS et coll en centrifugation lente, en gradient de ficoll triosil à la température de 18 °C. Le sang defibriné est mélangé à raison de 1 vol pour 3, à du sérum physiologique tamponné et deposé délicatement à la surface de la solution de ficoll triosil Après centrifugation de 20 mn à 400 g et 18 °C, les lymphocytes apparaissent à l interface plasma ficoll triosil, les différents éléments etant alors répartis de la manière suivante de bas en haut dans le tube globules rouges, granulocytes, plaquettes, dans le culot ficoll triosil, lymphocytes sous forme d un anneau, enfin plasma surnageant. Les lymphocytes sont alors prélevés à la pipette et resuspendus en liquide de Hanks.

Anticorps utilisés Nous avons utilisé un serum anti A de B de deux donneurs et titrant 1/512 (à dilution géométrique de raison 2 en eau physiologique) et un sérum anti B de A également de deux donneurs et titrant 1/350

Technique d'électrophorèse en phase liquide La mesure de la vitesse de migra tion d une particule colloidale dans un champ électrique est réalisée par observa tion microscopique dans une cellule de section rectangulaire ou circulaire (parfois à double voie) Pour éviter les effets électroosmotiques les mesures doivent etre réalisées en des points particuliers C'est ainsi que pour une cellule circulaire il faut se placer au point

$$r = \frac{\sqrt{2}}{2} R$$

(où R est le rayon de la cellule) alors que pour une cellule rectangulaire on se placera aux points définis par les formules de KOMAGATA

$$\left[y = \pm b \sqrt{1/3 \left(1 + \frac{384}{\pi^5 k}\right)} \right]$$

Nous avons d'ailleurs eu l'occasion de discuter la validité de ces formules [14]. Pour toutes les expériences décrites ci-dessous, nous avons utilisé un appareil d'électrophorèse appelé cytosphéromètre avec des électrodes à double bouchon de plâtre et une cellule rectangulaire [15].

Préparation des suspensions lymphocytaires. A partir des suspensions lymphocytaires en milieu de Hanks, nous avons préparé les suspensions de mesure. Celles-ci ont alors été préparées par mélange à volume égal de la suspension en milieu de Hanks avec, soit l'antisérum, soit le sérum d'un donneur de groupe AB dont on avait établi au préalable qu'il ne contenait ni anti H, ni anticorps antiérythrocytaires ou antilymphocytaires. L'utilisation d'un tel protocole était nécessaire, car la comparaison entre les résultats doit être réalisée à force ionique constante.

Résultats

Nous avons pris comme mobilité électrophorétique[2] de référence (Mo) la mobilité de lymphocytes de groupe O[+] suspendus dans un mélange milieu de Hanks + sérum AB. Ceci nous a permis de définir, dans un milieu donné, la mobilité électrophorétique relative (M_r) qui est le rapport entre la mobilité dans le milieu considéré et la mobilité de référence Mo.

Nous avons alors obtenu les résultats suivants: (a) le sérum AB est sans action significative sur la mobilité relative des lymphocytes A ou B (tab. I, colonnes 1 et 3), (b) les antisérums utilisés sont sans action sur la mobilité relative de lymphocytes de groupe O (6 expériences pour chaque antisérum), (c) la mobilité relative des lymphocytes en milieu Hanks + antisérum est toujours diminuée (11 suspensions pour le groupe A, et 7 suspensions pour le groupe B) (tab. I, colonnes 2 et 4).

Du point de vue statistique, nous pouvons considérer que nous avons deux échantillons identiques (les suspensions de lymphocytes A ou B) qui reçoivent deux traitements différents (mise en présence d'un sérum AB ou mise en presence d'un antisérum). On peut donc appliquer le test des séries appariées, c'est à dire tester, par rapport à l'hypothèse nulle, les différences de mobilité entre les lymphocytes (A ou B) suspendus dans du sérum AB et suspendus dans l'antisérum correspondant.

A l'aide des resultats du tableau I, nous pouvons alors conclure que les populations considérées sont distinctes avec un seuil de signification à 5%. C'est-à dire que l'antisérum a bien une action sur les lymphocytes

[2] La mobilité électrophorétique est définie comme étant le rapport entre la vitesse de migration et le champ électrique. Généralement on l'exprime en μ/sec/V/cm.

Tableau I Influence d'un sérum AB et d'antisérums anti A et anti B sur la mobilité électrophorétique relative de lymphocytes A et B (Mobilité de référence lymphocyte O+ en milieu Hanks + sérum AB)

Numéro d'expérience	*Mobilité relative* de lymphocytes B en présence de sérum AB	*Mobilité relative* de lymphocytes B en présence d'anti B	*Mobilité relative* de lymphocytes A en présence de sérum AB	*Mobilité relative* de lymphocytes A en présence d'anti-A
1	1,01	0,87	0,97	0,87
2	0,98	0,90	1,05	0,85
3	1,03	0,82	0,94	0,89
4	0,95	0,84	1,02	0,86
5	1,02	0,83	1,04	0,88
6	1,00	0,84	0,99	0,88
7	1,02	0,86	1,02	0,81
8			1,01	0,87
9			0,98	0,86
10			1,04	0,85
11			0,96	0,86
Moyenne	1,00±0,06	0,85±0,06	1,00±0,07	0,86±0,05

de groupe erythrocytaire correspondant. Comme nous avons travaillé à force ionique constante et que la seule difference entre les milieux de suspension est la présence ou l'absence de l'anticorps correspondant à l'antigène de même spécificité, supposé présent sur les lymphocytes, la signification des résultats met en évidence, de façon indirecte, la fixation des anticorps sur la membrane lymphocytaire et par suite la présence de l'antigène correspondant.

Conclusion

L'étude des variations de la mobilité électrophorétique de lymphocytes de groupes A ou B en présence d'anticorps anti-A et anti-B met en évidence une diminution de celle-ci, ce qui permet de mettre en évidence de façon indirecte la présence des antigènes correspondant sur la membrane lymphocytaire Il est certain que les résultats restent qualitatifs et qu'aucun calcul de dénombrement des sites n'est possible. Cependant, ces résultats sont conformes à ceux que nous avons déjà trouvés pour les hématies [16] et les plaquettes [14]

Résumé

Les auteurs étudient à l'aide de l'électrophorèse en phase liquide l action des anticorps anti A et anti B sur la charge de lymphocytes A, B ou O Ils constatent alors une diminution de mobilité électrophorétique lorsque l'anticorps et l antigène (supposé présent), de même spécificité sont mis en présence Comme la force ioni que reste constante au cours de l expérience, ces résultats permettent donc de mettre en évidence de façon indirecte l existence des antigènes du système ABO sur les lymphocytes humains

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Acta haemat. 47 140–145 (1972)

Androstane Therapy of Aplastic Anaemia

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Abstract. Androstane therapy in 34 new cases of aplastic anaemia together with the follow up in 33 cases previously reported is evaluated. The new cases were of the acquired variety and had a hypoplastic marrow. Remission rate was 50%.

Key Words
Androgen therapy
Androstane in aplastic anaemia
Aplastic anaemia
Methenolone
Oxymetholone

Evidence on the usefulness of different androstanes in the treatment of aplastic anaemia has been increasing in the past 10 years [1–6]. At present androstanes are considered as the agents of choice in the treatment of aplastic anaemia. However, there are still some controversial points in regard to androstane therapy, i.e., duration of remission, relative value of different androstanes, prognostic value of haemoglobin F levels, and degree of aplasia of the bone marrow.

Materials and Methods

The clinical material, 34 patients with acquired aplastic anaemia, 11 females and 23 males, was seen between October 1966 and September, 1970 in 2 hospitals: the Instituto Nacional de la Nutrición in Mexico City and the Hospital Civil Miguel Silva in Morelia, Mexico. All cases had normocytic normochromic anaemia, thrombocytopenia, leukopenia, neutropenia, and marked bone marrow hypocellularity with decreased or absent megakaryocytes. No splenomegaly, adenopathy, clinical or laboratory evidence of infectious, neoplastic or deficiency conditions were present. In addition, in 17 cases acid and sucrose hemolysis tests were performed with negative results in all instances.

The duration of illness to the start of androstane therapy was 2 months or less in 10 patients, 2.1–6 months in 12 subjects and 6.1–24 months in the others. Pre-

vious therapy consisted of prednisone (15–60 mg/day) for 1–2 months in 4 patients, and prednisone combined with different androstanes in another 5 (10–60 mg/day and 5–150 mg/day, respectively) for 12–34 days No response to previous therapy was observed

The compounds used by us were 17 β hydroxy 17α methyl 2 hydroxy methylene 5 α androstan 3-one (oxymetholone) in 32 patients and 17 β hydroxy 1 methyl 5-α androstan 1-en 3-one (methenolone) in 2 others. Both steroids were given by mouth, at daily doses of 1–1.5 mg/kg in 7 patients, 1 6–2 0 mg/kg in 16 cases and 2 1–3 mg/kg in 11 subjects

Results

Analysis of New Cases

Response to therapy Seven cases died within the first 2 months of therapy, and no response was observed in another 10 patients treated for 2–9 months The outcome of 2 of these cases is unknown, the other 8 died, two of them shortly after splenectomy, which was performed as a last resort in view of the failure of androstane therapy

The remaining 17 cases responded favorably As it has been usually observed [1–6], the response started with a rise in reticulocytes and stabilization in hemoglobin level These early signs were observed 2 weeks to 15 months after therapy was started (median 3 months), from then on hemoglobin rose slowly to normal levels and the number of platelets and neutrophils increased Simultaneously with haematological recovery, clinical manifestations, particularly the bleeding, decreased initially and eventually disappeared

Three patients relapsed when therapy was withdrawn One of them became pregnant and required two blood transfusions After delivery and without additional therapy, red cell values, platelets and neutrophils rose again to normal levels and have remained so for 15 months In the other 2 cases reinstitution of androstane therapy again induced a favorable response, in one the second remission continues 18 months after therapy withdrawal, whereas the other has required continuous maintenance treatment.

No relapse after discontinuation of androstanes was observed in the other 14 cases, though in 4 of them less than 6 months have elapsed since therapy was stopped

Prognostic factors (table I) The remission rate was higher in females than in males, and in the idiopathic variety than in the secondary one, but the differences were not significant ($p > 0.3$ in both instances) All

Table I Association of certain characteristics and the response to anabolic steroids

	Total	Remission	Remission rate, %
Sex			
Males	23	10	43
Females	11	7	64
Etiology			
Idiopathic	7	5	71
Secondary	27	12	44
Neutrophils/mm³			
Less than 500	13	0	0
More than 500	21	17	81
Reticulocytes %			
0 5 or less	16	4	25
More than 1 0	12	8	67
Haemoglobin F, mg/100 ml			
<100	7	4	
101–200	1	1	67
201–300	1	1	
301–400	2	0	40
>400	3	2	

cases with less than 500 neutrophils/mm³ failed to respond whereas a satisfactory response occurred in 17 of 21 with more than 500 neutrophils/mm³ ($p<0\ 001$) The influence of the reticulocyte per cent count was less marked

High levels of haemoglobin F were not associated with a favourable prognosis (table I)

Side effects Hoarseness and hirsutism were frequently observed in females and males younger than 15 years of age, but in no case were they intense enough to require stopping therapy or reducing dosage Two adults males after several months of oxymetholone therapy had mild galactorrhea which regressed without stopping the steroid Three patients complained of mild to moderate leg cramps

Clear abnormalities in liver function tests occurred in 4 patients while on therapy with oxymetholone and in 1 of the 2 under methenolone ad-

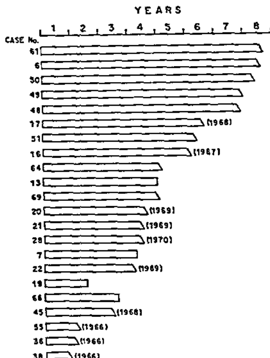


Fig 1 Follow up in 22 cases of aplastic anaemia with long lasting remissions after androstane therapy Non leveled bars indicate end of the remission due to acute leukemia (case 13) renal failure secondary to diabetic nephropathy (case 7) and a new episode of aplastic anaemia secondary to re-exposure to benzol (cases 19 and 66), these two patients responded again to oxymetholone therapy Beveled bars indicate patients who were in remission at the time of last consultation. If patient was not seen in 1971, the year of last consultation is given after the bar

ministration The liver changes seemed to be independent of androstane therapy and secondary to gallbladder disease in 1 case and septicemia in 2 patients In 2 other cases the liver alterations were mild and non progressive They disappeared shortly after therapy was stopped in one of them and reversed to normality in the other without changes in therapy

Follow-Up of the Cases Previously Reported

In 1969 [7] we reported 33 cases showing a remission after androstane therapy Two of them were lost soon after remission started and

another relapsed after re-exposure to DDT and died Information available on the remaining 30 cases is as follows The course in 22 cases with a permanent remission after the first therapeutic trial is graphically shown in figure 1 Another 8 relapsed after androstane withdrawal In 4, long-lasting remissions followed a second therapeutic trial Three cases relapsed whenever therapy was discontinued and one of them became refractory even to very large doses of androstanes (15 mg/kg/day of dromostanolone) and died

Comments

Results in the 34 new cases of the present series are in agreement with other reports [7–10] that the degree of bone marrow hypoplasia is not a significant prognostic factor in aplastic anaemia treated with androstanes, that there is a lag in the response to therapy, that females respond better than males, and that higher number of neutrophils and reticulocytes are associated with a better prognosis The remission rate observed in this series, constituted by cases with aplastic bone marrow, is comparable to the 53 4-percent obtained in over 600 cases with different degrees of bone marrow cellularity treated with androstanes [6]

A significant prognostic value in acquired aplastic anaemia has been ascribed to the level of haemoglobin F [11] We, however, have failed to confirm this observation as the remission rate was greater in our group of cases with lower levels The experience of WEINSTEIN [12, 13] is similar to ours mean haemoglobin levels were 214 and 417 mg/100 ml in 14 responding and 5 non-responding cases, respectively

The observation in this series of a higher remission rate in the idiopathic variety of the disease is contrary to what has been seen in larger series [1, 7] and probably is explained by the limited number of cases with such a variety included in this report

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Acta haemat 47 146-156 (1972)

Epsilon-Aminocaproic Acid for Synovectomy in Haemophilic Patients

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Abstract The study of coagulation and fibrinolysis in synovectomised haemophilic patients, treated by a new haemostatic combined therapy, is reported. This treatment is based on a combination of replacement therapy (fresh frozen plasma and/or cryoprecipitates) and ε aminocaproic acid the replacement therapy is continued for only 6 days beginning on the day of operation Our combined therapy scheme promotes a more efficient haemostasis than substitutive therapy alone and allows a considerable saving in plasma or plasma fractions

Key Words
ε-Aminocaproic acid
Haemophilia
Haemostasis
Joint surgery
Synovectomy

ε-Aminocaproic acid (EACA) has been used for many years in haemophilic patients for the prophylaxis of haemorrhages and during minor surgery such as dental extraction For extractions EACA has proved very useful when associated with other topical therapy [1, 6, 9, 15, 16] It has recently been pointed out that by administering EACA in doses of 0 4 g/kg/day for 6–8 days after dental extraction it is possible to avoid substitutive therapy with fresh frozen plasma or with cryoprecipitates [1] We have been using this form of therapy with good results for many years

As well as in minor surgery, EACA has proved to be very important in the major surgery of haemophiliacs, for instance, in synovectomy and other orthopaedic operations This has been amply demonstrated by STORTI *et al* [10, 11, 13] who first used synovectomy in the treatment of haemophilic haemarthrosis, at the same time indicating a new haemostatic treatment which has proved most effective in checking post-operative haemorrhage The treatment is based on a combination of substitu-

tive therapy and antifibrinolytic drugs, as follows (1) fresh frozen plasma, 18–20 ml/kg body weight, or an equivalent quantity of cryoprecipitates, for 6 days, beginning on the day of operation, (2) EACA 0.5–1 g/kg/day, orally or intravenously, for 4 weeks, (3) Frey's parotid inhibitor, 200,000–500,000 U into the cavity of the joint during operation

This treatment was applied in 50 synovectomies and 16 operations involving major orthopaedics arthrodesis, osteotendotomy, etc In no instance did bleeding cause any concern, though severe haemophilia, A or B type, was present in most cases The treatment was devised by STORTI *et al* [10, 11, 13] because their first 2 patients to undergo synovectomy bled profusely for 4 weeks, although substitutive therapy with 18 ml/kg/day was maintained throughout. It represents a considerable economy on the requirements of substitutive therapy as well as providing more effective haemostasis than by the other method which calls for high doses over an extended period of time

Results obtained from studies made on coagulation and fibrinolysis in some of the patients who underwent synovectomy and were treated by our method are reported below

Materials and Methods

Since April 1966 we have performed a total of 52 synovectomies on 43 patients, 37 with haemophilia A and 6 with haemophilia B Except in the first 2 cases the above-mentioned therapy was applied [illegible]

[illegible] logical study of fibrinolysis to be made on the fragment obtained.

As stated above after removing the synovia, 200 000–500,000 U of Frey's parotid inhibitor were introduced into the joint cavity EACA was administered during the subsequent days, preferably by the oral route, in single doses every 2 h, except in certain rare cases where gastrointestinal intolerance occurred.

Patients were subjected to clotting and fibrinolysis studies by sampling performed before replacement therapy, then again at the beginning of the operation and 12 h after the operation. For the next 4–6 days this examination was made daily before and after administering frozen plasma or cryoprecipitates.

Recalcification time and prothrombin consumption were measured by the usual methods. The koalin-activated partial thromboplastin time was measured [illegible]

[illegible] evaluations of the various phases of

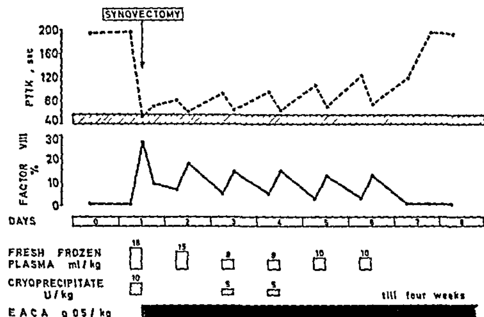


Fig 1 Partial thromboplastin time and factor VIII level in a patient before and after synovectomy Case No 38, haemophilia A, 13 years, 49 6 kg

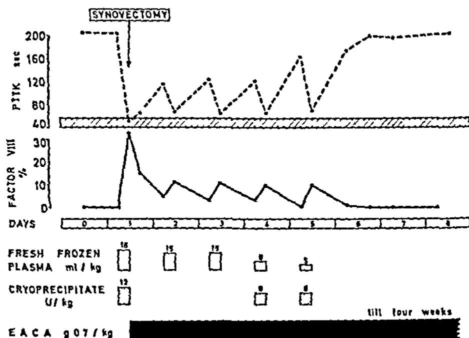


Fig 2 Partial thromboplastin time and factor VIII level in a patient before and after synovectomy Case No 41, haemophilia A, 18 years, 67 8 kg

fibrinolysis could be obtained To test the global fibrinolytic activity, the following methods were used the caseinolysis method of BRUHN *et al* [5], the fibrin plate method according to ASTRUP and MULLERTZ [3] the plasminogen free fibrin plate method according to BISHOP *et al* [4] The same group of methods was also used when employing both simple and streptokinase and urokinase activated euglobulin fractions

Antifibrinolytic activity was evaluated by taking the difference between the plasma fibrinolytic activity and the activated euglobulins activity

Results

In all patients subjected to synovectomy, the recalcification time, the partial thromboplastin time and the prothrombin consumption were found to be normal or only slightly changed at the beginning of the operation The antihaemophilic globulin content varied, however, from 25 to 40%

Figures 1 and 2 relate to 2 patients, cases No 38 and 41, which are quoted here as examples These figures show the pattern of partial thromboplastin time and factor VIII on operation day and on the days following In both cases the antihaemophilic globulin content remained at relatively low levels, with no bleeding The partial thromboplastin time showed changes corresponding to the factor VIII level variations

Figures 3 and 4 show the pattern for the post-operative phase of the first patient, operated on in April 1966, and of patient No 39, operated on in October 1970 In the first case (fig 3) EACA was not employed, and joint bleedings were observed notably on the 3rd, 8th and 16th days after operating, even though replacement therapy with fresh frozen plasma was continued No bleeding was observed in the other patient, who was treated with EACA, and for whom replacement therapy was discontinued after 6 days (fig 4)

In the 5 most recent cases, in which fibrinolysis was studied with the aid of all the above mentioned methods, the global lytic activity on urokinase-activated plasma, evaluated by the caseinolysis technique, was found in all cases to have decreased sharply after operating (fig. 5) This decrease, which showed values of 30–15%, whereas the values of normal subjects and of patients prior to operation ranged from 65 to 100%, lasted for about 72 h After this period levels of 50–60% were again recorded in all cases examined In the days following fibrinolytic activity remained at intermediate values

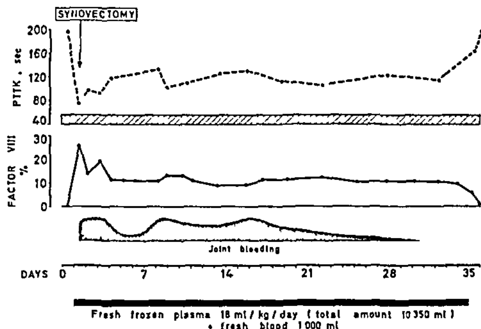


Fig 3 Post operative course in a patient treated with replacement therapy alone Case No 1, haemophilia A, 5 years, 17 0 kg

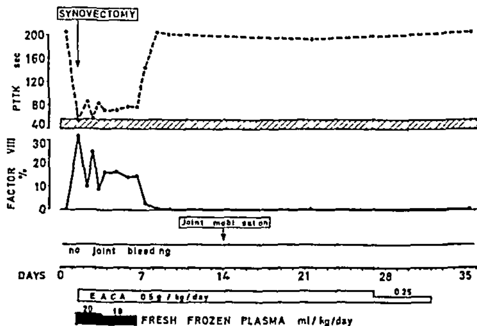


Fig 4 Post-operative course in a patient treated with replacement therapy and EACA Case No 39, haemophilia A, 19 years, 46.5 kg

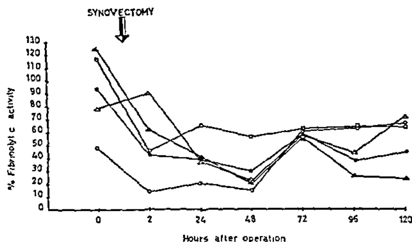


Fig 5 Global lytic activity of urokinase activated plasma in the 5 most recent cases subjected to synovectomy Fibrinolysis in plasma + UK.

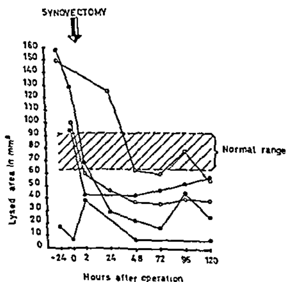


Fig 6 Study of streptokinase activated euglobulin fraction in the same 5 cases. Fibrinolytic activity in euglobulins + SK.

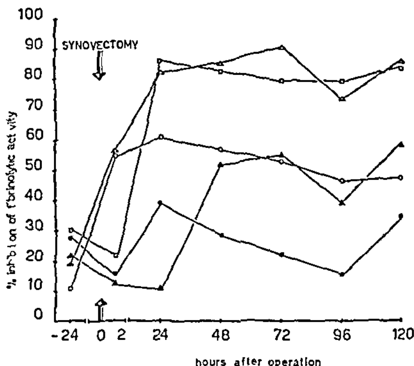


Fig 7 Antifibrinolytic activity in the same 5 cases Antifibrinolytic activity in haemophiliac s plasma

Studies of the streptokinase activated euglobulin fraction, carried out by the plasminogen free plate technique, showed behaviour similar to that of the global lytic activity (fig 6) Immediately after the operation activity diminished, and this fall continued until the 72nd h These val ues subsequently returned to intermediate levels in 4 of the 5 cases Only in one patient did the values remain markedly lower

On the other hand, studies of fibrinolytic activity in whole serum gave nil, and because of the large doses of EACA administered to pa tients, activation with streptokinase was difficult Antifibrinolytic activity in the same 5 cases showed specular behaviour when compared with the variations of fibrinolytic activity In fact, a clear increase was noted in antifibrinolytic activity, which reached its maximum within the first 72 h and remained at high levels during the days that followed (fig 7) With the other patients the various parameters of coagulation and fibri nolysis took a similar course

Table I Amount of replacement therapy required in synovectomy for the first 2 patients without EACA, and for the 6 most recent patients (mean values) with EACA

		Plasma		Cryoprecipitate	
		ml/patient	ml/kg/patient	U/patient	U/kg/patient
Case No 1					
Haemophilia A	17 kg	10 850	632.2	–	–
Case No 2					
Haemophilia B	26.5 kg	17 600	644 1	–	–
Cases No 38–43					
Haemophilia A. (mean value)	51.5 kg	4 400	85 7	958.3	18 66

As regards blood clotting the antihaemophilic globulin level, which was always higher than 25% at the time of operating, during the following days kept to values of between 5 and 15% Fibrinolysis was studied in 38 patients by the ASTRUP and MULLERTZ [3] method only, and the results from plasma and from simple and activated euglobulin fractions coincided very closely with those of patients studied by other recent techniques

After 15 days limb mobilisation was performed on all patients by stages and under the direction of a physiotherapist. Except in the case of one patient with a large necrosis at the distal end of the femur, replacement therapy was no longer necessary

Table I shows the plasma consumption, total and per kg, *in the first* 2 cases where EACA was not administered, the mean value of plasma consumption in the 6 recent cases (No 38–43) is also shown for comparison

Discussion

Our results, obtained from 43 patients subjected to synovectomy, demonstrate the great importance of the combined therapy reducing the need for a replacement supply of plasma, while at the same time promoting efficient haemostasis

In the first 2 cases, where no antifibrinolytic agents were used, the post-operative course was much more complex than in the other patients In the latter cases no bleeding episodes were observed, although replacement therapy was very much shorter and less strong.

Indirectly, the usefulness of EACA is also demonstrated by the levels of factor VIII, never high, which we found in the plasma of patients on the days following operation, there being no post-operative bleedings, as already stressed

The reason for using antifibrinolytic drugs was based on theoretical as well as experimental permises From the theoretical viewpoint, we consider that replacement therapy applied in the first days after operation is sufficient to cause a solid clot and to check post-operative bleeding at the outset During the healing of the wound, which in patients with haemophilia proceeds in the same manner as in the normal subject, the clot undergoes lysis and organisation by the newly formed granulation tissue which is rich in vessels It is at this stage that the antifibrinolytic agent, by blocking the clot lysis, enables provisional haemostasis to continue, at least until a cicatricial tissue without signs of blood has formed On the other hand, the walls of the newly formed vessels would seem to be the site of great fibrinolytic activity [14] The action of EACA can, therefore, be said to exercise a 'protective effect' on the primary clot, thus avoiding the need for a second haemostatic process which is obviously insufficient in these patients

The richness in vessels, which we observed in synovias of haemophilic patients in the first stages of haemophilic arthrosynovitis [12], may help to clarify the clinical observation that haemarthrosis tends to recur in the same joint It is possible that this abundance of vascular structures causes local stimulation of the fibrinolytic component, the importance of which has to be considered when treating haemarthrosis in this type of patient The importance of fibrinolysis as a cause of bleeding complications is also suggested by their delayed appearance, as has been observed by WALSH *et al* [16] These considerations should be regarded in the light of other experimental data recently noted by us [2] As is well known, a portion of the antihaemophilic globulin disappears from circulation very rapidly, in fact during the first few minutes after infusion, possibly because of its diffusion outside the vascular area [1] This extravascular distribution of protein is probably responsible for the fact that circulating factor VIII is of a consistently lower level than it is theoretically calculated to be after the infusion of plasma or other fractions

Our investigations showed that in patients treated with high doses of EACA the recovery of injected antihaemophilic globulin is higher than normal, sometimes reaching values corresponding to the theoretical ones [2] It is, therefore, possible that the extra vascular diffusion of the prote-

in is reduced, in terms of space by EACA, perhaps by modifying capillary permeability while favouring the pro-clotting moment in the haemostatic balance This observation may be of special importance in haemophiliacs who can be shown to have increased capillary permeability, at least so far as concerns the plasma clotting factors [17]

Moreover, the results reported in this paper demonstrate that antifibrinolytic activity in the serum of patients is so high that not even with streptokinase activation it is possible to achieve fibrinolytic activity

From these observations, when compared with replacement therapy alone, the use of EACA seems to us to be of primary importance in the post-operative treatment of patients with haemophilia EACA promotes more efficient haemostasis as well as a very considerable saving in plasma and/or plasma fractions, thus reducing the well known risks associated with their administration To these we would add obvious advantages of an economic and organisational kind

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Acta haemat. 47 157–163 (1972)

Determination of Iron in the Acid-Soluble Fraction of Human Erythrocytes

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Abstract In extracts of the acid-soluble fraction of human erythrocytes, iron exists in form of complexes with nucleotides, and during separation on Dowex I columns it was completely eluted in that form. The results indicate that about 50% of Fe present in the extracts formed complexes with ATP, and the remaining iron was eluted together with AMP, NADP and ADP. Small amounts of Fe added during the preparation of extracts were found in fractions of eluate containing AMP, NADP and ADP and slightly influenced the content of iron in the complex with ATP

Key Words
Erythrocyte fractions
Erythrocyte iron
Erythrocyte nucleotides

In a previous paper [4] we found that 20–40% of ATP present both in erythrocyte hemolysates and in the whole blood exist in the form of an iron complex. The estimation of Fe in the ATP fraction obtained by the method of MILLS and SUMMERS [6] using constant gradient elution with formic acid – ammonium formate system – demonstrated the presence of Fe in the descending part of the ATP peak. It was suggested that the main iron ATP complex corresponded to the formula $Fe(ATP)_2$

GOUCHER and TAYLOR [2] discovered the presence of an ATP-Fe complex in erythrocytes and investigated the formation of complexes of Fe^{+++} with ATP and other nucleotides *in vitro*. They stated that iron forms with ATP two stable compounds corresponding to the formulas Fe(ATP) and $Fe(ATP)_2$ and less stable complexes with AMP and ADP. They suggested that these complexes participate in some enzymatic reactions, e g Fe(ATP) in the presence of Mg^{++} may act as the phosphorylating factor in the reaction of yeast hexokinase. On the other hand,

HARRISON *et al* [3] reported on the existence of iron in many commercial ATP preparations, isolated from biological materials

In the present work we intended to determine the content of iron in the acid-soluble fraction of human erythrocytes and to exclude the possibility that complexes of iron with nucleotides may be artifacts formed during the preparation of extracts

Methods

We used human blood taken from the cubital vein by venipuncture from non selected blood donors

All reagents were of analytical grade and the solutions were prepared using bi distilled water

Laboratory glass before each use was dipped for 24 h in a mixture of concentrated HCl and H_2O (1 2), carefully washed in current water, distilled water, bi distilled water and dried

Chromatographic separation on Dowex 1 Extracts of the acid soluble fraction of erythrocytes were prepared according to the method of BARTLETT [1] and analyzed using the method of MILLS and SUMMERS [6] In the last method, a constant gradient elution from a glass column containing Dowex 1 X8 (formate, 200–400 mesh, resin bed 0 7×10 cm, flow rate 1 0 ml/min, fraction volume 3 ml) was used The required concentration gradients were obtained by a subsequent addition of 60 ml of 0.5 M formic acid, 150 ml of 4 M formic acid and 240 ml of 4 M formic acid-0 8 M ammonium formate into the mixing vessel containing initially 75 ml of water Absorbance at 260 nm on a Hungarian Spectromom 201' spectrophotometer and iron content of subsequent fractions were estimated

Determination of iron Iron was estimated by the method of RAMSAY [7] in the extracts of the acid soluble fraction of red cells before separation, in the effluent collected while putting extract on the column and in each sample obtained during constant gradient elution

2 ml of iron-containing solution, 2 ml of 0 1 M Na_2SO_3 and 2 ml of a 0 1 percent solution of 2 2'-dipyridyl in 3% CH_3COOH were thoroughly mixed in tubes and heated for 5 min in a boiling water bath During heating the complex of dipyridyl and Fe^{++} is formed After cooling absorbance measurements at 530 nm were read in the spectrophotocolorimeter Spekol (Carl Zeiss, GDR) using 1-cm glass absorption cells. Corresponding blanks were estimated parallel A stock solution of ferric ammonium sulfate (10 mg of Fe/100 ml) was prepared as follows 0 863 g of ferric ammonium sulfate was dissolved in a small volume of H_2O and after addition of 5 ml concentrated H_2SO_4 was filled up to 1 l with H_2O Iron standards containing 50 100 150 and 200 μg of iron/100 ml were obtained by dilution with water or with 4 M formic acid

The determination of the contents of nucleotides and Fe was also performed after the addition to red cells or to the extract of ferric ammonium sulfate solution (20 μmoles of Fe/100 ml of erythrocytes) during the preparation of extracts (table II)

Table I *Contents*[1] *of nucleotides*[2] and Fe in the acid-soluble fraction of human erythrocytes

No	AMP[2]	ADP	ATP	NAD	NADP	Percentage ATP in Fe (ATP)[3]	Percentage Fe in complex with ATP	Fe in ATP fraction	Fe in AMP, NADP and ADP fractions	Sum of Fe in the fractions	Content of Fe in extract	Amount of Fe in the effluent received while putting extract on the column	Fe retained in the column
1	1.17	47.1	121.3	5.1	5.0	20.8	45.5	12.6	15.1	27.7	26.4	0.55	25.85
2	1.85	33.1	116.2	8.6	5.1	22.3	47.8	13.0	14.2	27.2	29.7	0.60	29.10
3	2.39	33.5	153.0	7.7	4.5	15.7	55.4	12.0	9.7	21.7	20.0	1.20	18.80
4	1.69	26.8	116.3	11.1	2.7	21.8	57.5	12.7	9.4	22.1	20.8	0.20	20.60
5	3.35	30.6	124.0	9.1	4.1	20.8	63.7	12.9	7.4	20.3	20.3	0.34	19.96

[1] Contents of nucleotides and Fe expressed in µmoles/100 ml of erythrocytes
[2] E_{mM} at 260 nm used AMP, ADP and ATP, 13.5, NAD and NADP, 18.3
[3] The values given represent the mean average of 3 determinations of the same blood

Table II Contents[1] of nucleotides[2] and Fe in the acid-soluble fraction of human erythrocytes after the addition of 20 μmoles of Fe/100 ml of erythrocytes

	AMP[2]	ADP	ATP	NAD	NADP	Percentage ATP in Fe (ATP)[3]	Fe in ATP fraction	Fe in AMP, NADP and ADP fractions	Content of Fe in extract	Amount of Fe in the effluent received while putting extract on the column	Fe retained in the column
Control	3 35	30 6	124 0	9 1	4 1						
Red cells + Fe	2.44	39 5	129.2	6 93	4 1	20 8	12 88	7 42	20 3	0 34	19 96
Extract + Fe	2.18	41 5	122.4	6 96	3 8	21 6	13 96	28 06	42.4	0 38	42.02
						20 9	12.78	24 85	37 3	0 32	37 32

[1] Contents of nucleotides and Fe expressed in μmoles/100 ml of erythrocytes
[2] E_{mM} at 260 nm used AMP, ADP and ATP, 13 5, NAD and NADP, 18 3
[3] The values given represent the mean average of three determinations of the same blood

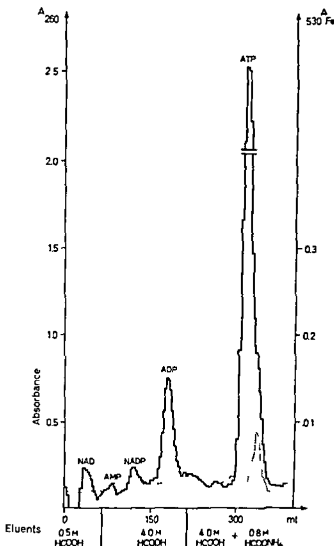


Fig. 1 Separation on Dowex I column by Mills's method using constant gradient elution with formic acid formate system —— A_{260} ...... $A_{530\ Fe}$

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The role of the described complexes remains unexplained. Free iron has toxic properties and for that reason iron complexes with organic compounds are investigated. Our results confirm the existence of complexes of Fe with nucleotides in human erythrocytes. Lately LEYKO *et al*

[5] observed a decrease of iron in ATP fraction of red cells in sideropenic anemia and it seems possible that the described complexes may play some role in the erythrocyte metabolism

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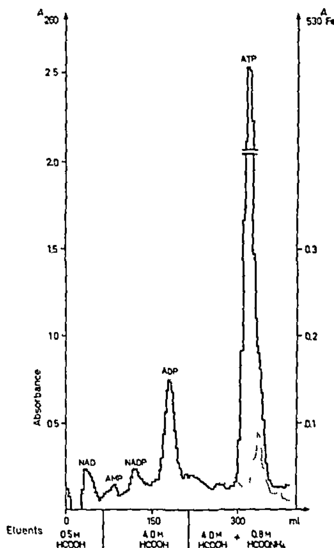


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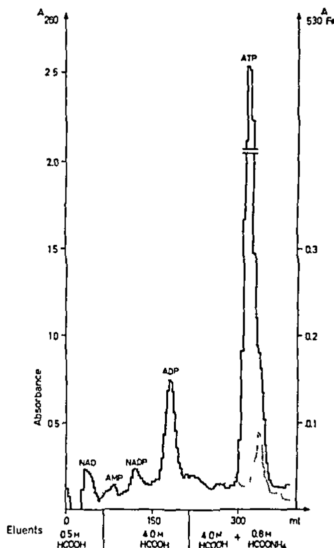


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Acta haemat 47 164–167 (1972)

Erythrocyte Content of Free Protoporphyrin in Thalassaemic Syndromes

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Abstract In a series of 31 patients with heterozygous and 20 patients with homozygous β thalassaemia the erythrocyte protoporphyrin content was found constantly raised The difference from normal values was statistically significant

Key Words
Thalassaemia
Erythrocyte protoporphyrin
Haem biosynthesis

The erythrocyte content of free protoporphyrin does normally not exceed 40 μg/100 ml [1] In various haematological disorders this level has been found considerably increased Thus, in iron deficiency anaemia various authors [6, 11, 13] found the erythrocyte protoporphyrin (EP) levels constantly above normal values These results were further confirmed by Dagg *et al* [3] who, in addition, found similar increase in a group of sideropenic patients without overt iron deficiency anaemia Similarly, high EP levels were found in the anaemia of chronic infection [1] and in the anaemia accompanying rheumatoid arthritis [5, 10] In the acquired type of sideroblastic anaemia the EP level was almost always raised [8]

In the thalassaemias measurements seem to indicate that the EP level is rather high, but so far the number of patients studied has been relatively small In view of this it has been deemed worth while to study the EP content in a large series of thalassaemic patients

Material and Methods

The subjects studied were as follows 20 haematologically normal subjects with Hb levels above 14 g/100 ml normal levels of serum Fe and a saturation of the

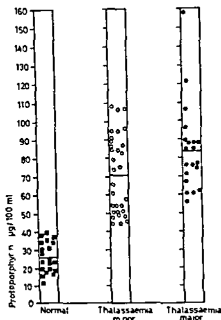


Fig 1 Free protoporphyrin content of erythrocytes.

total iron binding capacity greater than 16%. 31 patients with a haematological picture typical of heterozygous β thalassaemia (minor), 20 patients with homozygous β thalassaemia (major)

Standard haematological determinations were performed as described by DACIE and LEWIS [2] EP was determined by the method of RIMINGTON *et al* [16] Serum iron was measured by the method of PETERS *et al* [12] For the estimation of the saturation of the total binding capacity the method of RAMSAY [14] was used

Results

The mean value for EP in normal subjects was 25 6 ± SD 9 54 µg/100 ml erythrocytes and the range was 11–39 µg/100 ml The mean value obtained in patients with heterozygous β-thalassaemia was 70 4 ± SD 22 19 µg/100 ml erythrocytes (range 44–108 µg) This is significantly different from the normal group ($p<0\ 0005$) In the group of patients with homozygous β thalassaemia the mean value was 83 3 ± SD 23 42 µg/100 ml erythrocytes (range 55–156 µg) This is significantly

different from the normal subjects ($p<0.0005$). The difference of the EP content between the heterozygous and the homozygous patients is lower, but statistically significant ($p<0.05$). Figure 1 illustrates the results.

Discussion

The present observations indicate that in thalassaemic syndromes the EP content is increased. The literature on the subject is rather limited. Thus WATSON [19] found normal EP levels in a case of thalassaemia major. SCHWARTZ, TIENE *et al.* [17] in 10 cases of thalassaemia major found increased EP levels varying from 96 to 168 μg/100 ml of erythrocytes. In 3 cases of thalassaemia major and 2 cases of thalassaemia minor GRINSTEIN *et al.* [4] found values varying from 43 to 96 μg/100 ml of erythrocytes. In the 10 cases of thalassaemia minor examined by LÜDIN [9] the EP values varied from 60 to 438 μg/100 ml. STURGEON *et al.* [18] examined 7 cases of thalassaemia major and 9 cases of thalassaemia minor. The EP values varied from 19 to 110 and from 22 to 75 μg/100 ml in the two groups respectively. Finally, KREIMER-BIRNBAUM and BANNERMAN [7] in a study on the interrelationship of pyrrole and globin metabolism in β thalassaemia quote that the EP content of a patient with heterozygous β thalassaemia varied from 35 to 85 μg with a mean value of 52.5 μg/100 ml of erythrocytes.

The elevation of EP in patients with β thalassaemia might be explained by the well known fact that although the thalassaemias are primarily disorders of globin synthesis, there is also impairment of haem biosynthesis. As RIMINGTON [15] points out the synthesis of haem and globin are closely linked.

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Acta haemat 47 168–174 (1972)

Virusreplikation in unreifen granulopoetischen Zellen bei Mäusen mit Rauscher-Leukämie[1]

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Abstract In Balb/c mice with Rauscher leukemia virus particles were demonstrated in granulopoietic cells after hypertransfusion of the animals So it is evident that no specificity of the Rauscher virus for erythropoietic cells can exist

Key Words
Leukemia virus
Murine erythroleukemia
Rauscher leukemia

Die virusinduzierte Rauscher-Leukämie wurde bisher überwiegend als Erythroleukämie beschrieben [2, 3, 6] Elektronenmikroskopisch gelang der Virusnachweis in den verschiedensten Zellsystemen, besonders fiel jedoch die Virusreplikation in den Megakaryozyten und in den Erythroblasten, vor allem denen in der Milz, auf [1, 8, 10] Wir wollten jetzt wissen, ob sich morphologisch Beziehungen des Virus zur Granulopoese im Knochenmark nachweisen lassen, da wir bei genauerem Studium der Hämopoese infizierter Tiere lichtmikroskopisch dort auch Veränderungen feststellen konnten Für die elektronenmikroskopische Untersuchung erschien uns die Unterdrückung der Erythropoese durch Hypertransfusion der Tiere nach Entwicklung der Leukämie besonders geeignet, da dann im Knochenmark eine weitgehend reine granulopoetische Zellpopulation beobachtet werden kann [5]

Material und Methode

Tierversuche In einem ersten Versuch wurden 20–22 g schwere weibliche Balb/c Mäuse (Gl Bomholtgard Ry Dänemark) mit dem Virus infiziert und nach 17

[1] Mit Unterstützung der Deutschen Forschungsgemeinschaft

Tagen untersucht (Milzgewicht 2,5 g, periphere Zellzahl 80 000 kernhaltige Zellen/mm^3, davon 60% Erythroblasten, HK 30%).

Im Hauptversuch wurden 23 Tiere infiziert. Nach 9 Tagen, nachdem das mittlere Milzgewicht bei Kontrollen 1,4 g (n=9) erreicht hatte, erhielten die Tiere an 2 aufeinanderfolgenden Tagen je 0,8 ml einer gewaschenen isologen Erythrozytensuspension i. p. 16 Tage nach der Infektion wurden sie zusammen mit einer weiteren unbehandelten virusinfizierten Kontrollgruppe (n jeweils 7) getötet.

Die hämatologischen Daten waren dann wie folgt (in Klammern jeweils die Mittelwerte der nicht hypertransfundierten Kontrolltiere): HK 65% (30%), periphere Erythroblastenzahl 0/mm^3 (86 600/mm^3), Erythrozyten 13,4×10^6/mm^3 (5,0×10^6/mm^3), Retikulozyten 0–2‰ (80–200‰), mittleres Milzgewicht 1,6 g (3,5 g). Weitere Details der hämatologischen Befunde siehe [5]. Intensive elektronenmikroskopische Untersuchungen wurden an 2 Tieren mit einem Hämatokrit von 68 bzw. 67% und 1‰ Retikulozyten durchgeführt. Im Milzausstrich dieser Tiere wurden nur vereinzelt kleine Erythroblasten gefunden, im Knochenmark lag ihre Häufigkeit bei nur noch etwa 1% aller kernhaltigen Zellen.

Elektronenmikroskopie. Fixation in 3,5%igem Glutaraldehyd, gelöst in 0,1 M Phosphatpuffer, End-pH 7,2, Fixationsdauer 1 h. Nach der Fixation wurde das Gewebe 4mal 30 min in der Pufferlösung gespült. Die Nachfixierung erfolgte in 1%iger OsO_4 für 2 h. Dehydrierung in aufsteigender Acetonreihe. Das in Araldit eingebettete Material wurde mit Uranylacetat und Bleicitrat [7] nachkontrastiert. Zur Kontrolle wurden Dickschnitte von 1–2 μm im Phasenkontrastmikroskop untersucht. Die Herstellung der Ultradünnschnitte erfolgte mit dem LKB Ultratom III, sie wurden auf Formvar befilmte Kupfergrids aufgefangen. Zur elektronenmikroskopischen Untersuchung benutzten wir das Siemens Elmiskop Ia.

Befunde

Im Knochenmark der lediglich virusinfizierten Tiere fanden wir zahlreiche Viren aller Reifungsstadien, vor allem in den Megakaryozyten und auch in den Erythroblasten. In den relativ wenigen Zellen der granulopoetischen Zellreihe konnten wir keine Zeichen einer Virusentstehung beobachten.

Im Knochenmark der hypertransfundierten Tiere wurden elektronenmikroskopisch keine sicheren erythropoetischen Zellen gefunden. Die Megakaryozyten zeigten eine Virusreplikation wie bei den unbehandelten Tieren. Alle Stadien der Virusreifung konnten hier jetzt aber auch an den Zellen der granulopoetischen Reihe gesehen werden. Dabei fielen ganz selten auch völlig undifferenzierte Zellen mit grossem Nucleolus ohne Granula auf, die an ihrer Zellmembran knospende Viruspartikel aufwiesen (Abb. 1). Dieser für die Virusreplikation charakteristische Vorgang fand sich auch bei zahlreichen Myeloblasten, Promyelozyten



Abb. 1 *a* Undifferenzierte Zelle, Virusreplikation an der Zellmembran (→) × 23 000 *b* Stärkere Vergrösserung von Abbildung *1a*



Abb. 2 Myeloblast. An der Zellmembran (→) Virusreplikation × 18 000



Abb. 3 Promyelocyt. Virusreplikation (→). × 25 000

und Myelozyten, also den noch proliferierenden unreifen Stufen der Granulopoese (Abb 2, 3) Bei den reiferen Zellen der Granulopoese bis hin zum neutrophilen segmentkernigen Granulozyten gelang der Virusnachweis jedoch nicht, obwohl zahlreiche Zellen untersucht werden konnten Zusätzlich wurden knospende Viruspartikel ganz vereinzelt auch in Endothelzellen gesehen

Diskussion

Die Unterdrückung der Erythropoese durch Hypertransfusion der Mäuse einige Tage vor der Untersuchung des Knochenmarks hat sich in unserem Versuch als geeignet erwiesen, die Beziehung von Virusreplikation und Granulopoese aufzudecken Den negativen Nachweis des Virus in granulopoetischen Zellen bei «Normaltieren» 17 Tage nach Virusinfektion möchten wir vor allem auf die quantitativ geringere Zellpopulation zurückführen, die bei diesen Tieren untersucht werden konnte Die Hypertransfusion hebt diesen Nachteil auf

Der von uns geführte Nachweis der Virusreplikation in granulopoetischen Zellen nach dem Kunstgriff der Hypertransfusion spricht gegen die weitgehend angenommene Spezifität des Rauscher Virus für die erythropoetische Zellreihe und die Megakaryozyten, in denen sich freilich alle Leukämieviren bevorzugt nachweisen lassen [Übersicht bei 9] Die bei der Rauscher-Leukämie in Balb c Tieren von uns gefundene relative Vermehrung granulopoetischer Vorstufen mit einer pathologischen Reifeverteilung, die durch die Hypertransfusion nicht beeinflusst wird [5], geht also mit einer Virusreplikation in diesen Zellen einher

Bemerkenswert erscheint uns noch das Fehlen von Viren bei den ausgereiften Granulozyten Das prinzipiell gleiche Phänomen haben wir früher auch an der Erythropoese in der Milz nachweisen können, wo die reifsten Erythroblasten ebenfalls keine Viren erkennen liessen [8] Ob sich diese virusfreien Zellen von ebenfalls virusfreien Vorstufen ableiten oder ob die Ausreifung eine weitere Virusvermehrung nicht mehr zulässt, kann hier nicht entschieden werden Der Virusnachweis an Zellen der Erythropoese und der Megakaryozytopoese entspricht den in der Literatur wiederholt mitgeteilten Befunden

Die Bedeutung der hier mitgeteilten Beobachtung liegt unseres Erachtens darin, dass unter geeigneten Bedingungen die Replikation des Rauscher-Virus in allen 3 hämopoetischen Zellinien des Knochenmarks nachgewiesen werden kann Wir sehen darin eine Stütze für unsere an



Abb. 3 Promyelocyt Virusreplikation (→) × 25 000

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anderer Stelle dargestellte Vermutung [4] dass das Virus an den gemeinsamen undifferenzierten Vorläuferzellen angreifen konnte die dann unter laufender Virusreplikation in die Erythropoese und Megakaryozytopoese und eben auch in die Granulopoese hineinreifen

Zusammenfassung

An Balb/c Mausen mit Rauscher Leukämie wurden nach Hypertransfusion elektronenmikroskopisch Viruspartikel an der Zellmembran unreifer granulopoeti scher Zellen gefunden Damit ist nachgewiesen dass keine Spezifität des Rauscher Virus für die Zellen der Erythropoese besteht

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Acta haemat 47 175–181 (1972)

Congenital Methemoglobinemia due to Diaphorase Deficiency

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Abstract A case of congenital methemoglobinemia due to diaphorase deficiency is presented Recessive inheritance of this disorder was documented by a family study In addition to congenital methemoglobinemia the propositus was mentally retarded Although his mother and a sibling were mentally retarded his siblings methemoglobin diaphorase activity was found to be normal and his mother s at the heterozygote level

Key Words
Diaphorase deficiency
Hereditary methemoglobinemia
Mental retardation

In our previous publication a family with hereditary methemoglobinemic cyanosis due to diaphorase deficiency in 3 successive generations was described [1] In this communication, a family with hereditary mental retardation will be presented one of whose 2 living children also had congenital cyanosis due to diaphorase deficiency

Cas Report

O P (fig 1 III 1), a 5 year-old Turk [illegible] was referred to Hacettepe Children s Hospital because of cyanosis and me[illegible] retardation Born when his mother was 25 and his father 33 years old he wa[illegible] result of an uneventful pregnancy and delivery Cyanosis was noted at birth [illegible] the cord blood was described as dark He did not have jaundice in the n[illegible] period and his cyanosis became less prominent after 3 months of age D[illegible] ieved the patient had congenital heart disease and he was referred to this [illegible] for evaluation of this diagnosis The parents were first cousins and [illegible] siblings had died in the neonatal period As can be seen from his family tree [illegible] mother was mildly mental

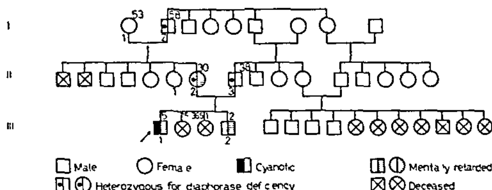


Fig 1 Pedigree of family with methemoglobinemia due to diaphorase deficiency The arrow indicate the propositus The numbers over the signs indicate the present age or the age at death

ly retarded, while his only living sibling was markedly so, however, only the propositus was cyanotic

On examination the child was found to have slate gray cyanosis of the lips and fingers, and was microcephalic (head circumference 48 cm) His height was 99 cm and weight 16 kg, and he was markedly mentally retarded The eye ground was normal, and there were no neurologic abnormalities other than mental retardation The lungs were clear, the heart sounds normal and there was no clubbing of the fingers Skull X ray was non contributory Hb 12 5 g%, hematocrit 41% and leukocyte count 8,400/mm³ with a normal differential Protein bound iodine was also within normal limits (3 8 γ/100 ml) The ferric chloride test for phenylketonuria was negative

Methods

The methemoglobin and fetal hemoglobin (HbF) concentrations were determined by the methods of EVELYN and MALLOY [2] and SINGER [3], respectively Erythrocyte reduced glutathione (GSH) glutathione stability, glucose-6-phosphate dehydrogenase and NADH-dependent methemoglobin reductase (diaphorase) activity determinations were performed by the methods of BEUTLER [4] GRUNET and PHILLIPS as modified by BEUTLER [5] ZINKHAM [6], and SCOTT with ROSS's modification [7] successively Starch gel electrophoresis was performed according to SMITHIES [8] and agar gel according to ROBINSON *et al* [9] The hemoglobin level was determined as cyanmethemoglobin For spectroscopic studies, Beckman's DU spectrophotometer was used Benzoquinone acetic acid in the urine was tested by FISHBERG's method [10]

Results

The concentrations of HbF, methemoglobin, the values of GSH, glutathione stability and the activities of G-6-PD and methemoglobin diaphorase in the propositus and members of his immediate family are shown in table I

Oxy-, meth- and cyanmethemoglobin electrophoresis on starch gel at pH 7 (phosphate buffer) in the control and the propositus did not reveal any difference Neither the patient's starch gel at pH 8 6 (tris-citric acid), nor agar gel electrophoresis at pH 6 45 (citrate phosphate) showed any abnormal hemoglobin component, and no benzoquinone acetic acid was found in the urine

The absorption spectra were obtained with a dilution of the propositus' hemolyzate 1 20 at pH 6.5 (M/15 phosphate buffer) By the addition of sodium cyanide, the peak around 630 disappeared and at that time there was no spectral difference between the control's cyanmethemoglobin A and that of the patient. The latter's methemoglobin level was redetermined following methylene blue treatment (2 mg/kg) by mouth, and was found to be 0 54%

Comments

The methemoglobin level was markedly increased in the propositus, and NADH *methemoglobin diaphorase activity was not found in his* erythrocytes His methemoglobin level became normal following methylene blue treatment The above laboratory findings indicate that this pa-

Table I Results of laboratory studies

	Age	MetHb, % (<1)	HbF % (<2)	G-6-PD, (110-165)	RBC GSH, mg % 0 h	2 h	NADH diaphorase $\Delta mOD \times 10^4 = \mu$ (29–60)[1]
Ö P (III 1) 5 propositus	5	22.5	1	173	109	112	0
A P (II 2)	30	0 7	1.2	113	164	122	18
I P (II 3)	38	0 7	3	112	139	103	20
Ö P (III 2)	2	0 6	3	131	205	95	48
I (I 2)	58	0 47	–	110	117	92	20

[1] Numbers in parenthesis indicate our normal values

tient had methemoglobinemic cyanosis due to NADH-dependent diaphorase deficiency The heterozygous level of this enzyme's activity in the parents was compatible with the autosomal recessive inheritance of this molecular disorder [11–18] CODOUNIS [19], however, has interpreted the results of his patient's family studies as indicating a dominant mode of inheritance Re examination of one affected member from each of his original families revealed a severe deficiency of NADH-methemoglobin reductase activity [20] Dominant inheritance could not be rejected in our previous family with diaphorase deficiency either [1]

BLOOM and ZARKOWSKY [21] have shown heterogeneity of the enzymatic defect in congenital methemoglobinemia cases In the light of this finding, different modes of inheritance should be re-evaluated

The coexistence of mental retardation with congenital methemoglobinemic cyanosis due to diaphorase deficiency does not seem to be a chance occurrence [11, 12] A total of 29 patients with varying degrees of mental retardation associated with this kind of cyanosis in at least 19 different families had been collected [12]

Although in some cases the interaction of hypoxia with neonatal jaundice was postulated for the mental deficiency [22], the latter does not correlate with methemoglobin concentration, nor is it observed in cases of hereditary methemoglobinemia with HbM Therefore this hypothesis cannot be accepted without reservation [12–14, 23] The possible role of hypocalcemia to the development of neurological disorders was discussed in a recent paper by LO *et al* [24], but no suggested evidence of this combination was mentioned in the previous cases The second case of FIALKOW *et al* [22] looked normal in early infancy, but developed mental retardation later [12] The ultimate mental retardation of this patient could hardly be explained by hypocalcemia either

Although FIALKOW *et al* [22] did not find decreased activity of NADH-diaphorase of the white blood cells in their diaphorase-deficient patients with mental retardation, KAPLAN and HANZLICKOVA LEROUX [25] recently suggested its absence in such cases, but not in patients without mental retardation Since our patient lives far from Ankara, no blood could be obtained to explore this discrepancy in our case

Since NADH-dependent diaphorase has a reduction capacity of methemoglobin of over 70% [26], about one third of the normal capacity was lacking in the patient's parents and maternal grandfather, who were heterozygous by assay for this enzyme activity Heterozygosity does not usually cause significant methemoglobinemia, but could contri-

bute to increased susceptibility to methemoglobin producing drugs and chemicals, which has recently been reported in connection with malarial chemoprophylaxis in Vietnam [27]

Other causes of congenital methemoglobinemias were also checked in our patient. No abnormal hemoglobin could be detected on starch gel (at pH 8 6) and agar gel (at pH 6 4) hemoglobin electrophoresis The HbM group hemoglobins showed unique separation in the methemoglobin form from HbA on electrophoresis at pH 7 [23], which did not disclose any fraction in the propositus or his parents' hemolysates Erythrocyte GSH and glutathione stability of the patient were found to be normal, therefore he did not show the chemical evidence of the case reported by TOWNES *et al* [28, 29] Since benzoquinone acetic acid in the urine was not found, this type of methemoglobinemic cyanosis [10], possibly due to a metabolic defect, could also be ruled out.

NADPH methemoglobin reductase is another enzyme which could be effective for methemoglobin reduction, 6% without methylene blue, and more effectively with this substance [26] This enzyme activity has been assayed in at least 2 cases of congenital methemoglobinemia with diaphorase deficiency, and was found to be normal [30] In one report, a hereditary deficiency of NADPH methemoglobin reductase activity was suggested as the cause of cyanosis, but the patient's methemoglobinemia responded to the administration of methylene blue [31]

In our case, methemoglobinemic cyanosis with NADH methemoglobin diaphorase deficiency was combined with mental retardation Although there is a striking number of patients with mental retardation and this kind of methemoglobinemia, our patient's mother and brother also had retardation without methemoglobinemia, which makes the genetic relations more difficult to explain in this family However, in at least one other family other members had some degree of mental retardation without cyanosis [13, 22] The occurrence of this combination could very well be genetically related as other cases in the literature [11–13, 22, 24] or else the 2 conditions may be coincidental in this family

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Acta haemat. 47 182-191 (1972)

Von Willebrand's Disease: Platelet Nucleotide Alterations in a Case with Marked Platelet Adhesiveness and Aggregation Defects

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Abstract. A case of von Willebrand's disease with marked platelet defects is reported. In comparison with normal control subjects, the patient's platelets have presented low ADP and AMP levels. Six hours after a normal human plasma transfusion a transitory correction of platelet aggregation by ADP 10^{-4}M and a strong rise of all platelet nucleotides have been observed in addition to the increase of AHF activity and the partial normalization of the prolonged bleeding time. These data support the hypothesis that the temporary correction of the ADP-dependent aggregation has been due to an active synthesis of platelet nucleotides under the influence of one or more unknown factors contained in the transfused plasma.

Key Words
Angiohemophilia
Platelet function
Platelet nucleotides
Von Willebrand's disease

Von Willebrand's disease is an autosomal, frequently dominant, hereditary disorder in which the bleeding time is prolonged and the AHF activity (factor VIII) is decreased [1-5]. The AHF defect in von Willebrand's disease differs from that of hemophilia A, since the marked increase of factor VIII concentration after normal plasma, some plasmatic fractions and serum transfusions is probably due to an active synthesis of AHF [6-12]. Furthermore, the AHF increase may be obtained with hemophilic fraction 1-0 [3-5, 13, 14].

As stated by NILSSON *et al.* [3-5, 13, 14], the treatment of von Willebrand's disease with plasma fraction 1-0 shortens the prolonged bleeding time, since this fraction, in addition to AHF and fibrinogen, also contains another unknown factor, the so-called Willebrand's factor, active in the temporary correction of this alteration. These data have been

confirmed by CORNU *et al* [15] and by VAN CREVELD and MOCHTAR [16]

A conflicting area in the pathogenesis of this disease is the role that one or more platelet defects have on the abnormal bleeding time NILSSON *et al* [3–5] did not find any alteration of many platelet properties Moreover, they stated the inefficacy of normal platelet transfusions on the Duke's time These data have been recently reviewed and confirmed by CRONBERG *et al* [17]

On the contrary, platelet adhesiveness and aggregation have been found altered by many authors who have demonstrated an impaired adhesiveness to the glass [10, 18–21] and *in vivo* with the BORCHGREVINK method [22–26] in addition to a low aggregation by ADP [27] Moreover CORNU *et al* [7] BORCHGREVINK *et al* [24] ZUCKER [18] and SALZMAN and BRITTEN [21] have observed that normal plasma *in vivo* and *in vitro* restored in their patients a normal platelet adhesiveness

In 1963, CAEN and COUSIN [28, 29], in 7 cases of von Willebrand's disease found a high platelet ATP content and a normal rate of ADP with an elevated ATP/ADP ratio while their patients with Glanzmann's thrombasthenia belonged to 2 groups cases with an impaired platelet glycolysis and consequently low ATP levels and those without glycolytic anomalies, with low rates of platelet ADP Although the significance of these results is still unknown, the platelet nucleotide alterations found by CAEN and COUSIN may have a fundamental role in the impaired adhesiveness and aggregation in some cases of von Willebrand's disease.

The patient herein described has presented, besides a low AHF level and a prolonged bleeding time, a low platelet adhesiveness to glass and aggregation by ADP Moreover, normal human plasma transfusions have determined, after 6 h, a temporary reduction of the Duke's time and a correction of platelet aggregation Researches on platelet nucleotides, before and at some intervals after a human plasma transfusion, have shown interesting results which are reported and discussed in this work.

Methods

Standard methods [30] were used for hematological studies. Coagulation and hemostasis investigations were performed with the methods listed in table I.

Platelet nucleotide determinations Venous blood of 5 fasting normal donors ranging between 20 and 40 years and of our patient was obtained from an antecubital vein in plastic syringes and anticoagulated in plastic test tubes by one tenth volume of 3.8% sodium citrate. The blood specimens were centrifuged at +4 °C for 15 min at 900 rpm for the separation of the platelet rich plasma (PRP) After having discarded 4 ml of PRP the platelet poor plasma (PPP) was obtained with a centrifugation at +4 °C for 20 min at 3 000 rpm. The PRP platelet counts tested with the direct PALUMBO and DONI method [31] ranged between 200 000 and

Table I Studies on coagulation and hemostasis

	Patient	Normal values	Methods
Platelet count/mm³	290 000	200 000–400 000	PALUMBO and DINI [31]
Platelet morphology	normal		blood smears
Bleeding time, min	>20	≤5	Duke
Tourniquet test	positive (++++)		
Clotting time, min	20	≤6	Lee-White
Clot retraction, %	85	80–100	Fonio
Howell, sec	420	120–180	Howell
Partial thromboplastin time (PTT) sec	132	40–60	PROCTOR and RAPAPORT [32]
Prothrombin time, sec	12	12	
Prothrombin consumption %	64	≥90	AGGELER *et al* [33]
Fibrinogen, mg%	470	200–400	RATNOFF and MENZIE [34]
Thromboelastography, mm	r 24, k 24 ma 30 ε 43	r 8–12, k 7–11, ma 55–60	Hartert
Thromboplastin generation test			Biggs and Douglas
With patient's plasma	8 min 28 sec	8 min 8–12 sec	
With patient's serum	8 min 12 sec	8 min 8–13 sec	
With patient's platelets	8 min 14 sec	8 min 11–15 sec	
AHF activity, %	23	50–140	RAPAPORT *et al* [35]
Platelet adhesiveness, %	4	20–40	HARDISTY *et al* [36]
Platelet aggregation by ADP 10^{-4} M (final concentration), %	14	30–50	BORN [37]

400 000 mm³ and no erythrocytes or leukocytes were detectable The platelet counts on PPP did not reveal in any case the presence of blood cells.

3 ml of both PRP and PPP were homogenized with 3 ml of ice cold 0 6 N perchlorid acid and the precipitated proteins removed by centrifugation ATP, ADP and AMP assays on the supernatants of both PRP and PPP were performed using Biochemica test kits TC-J 15979 and TC K 15980 (C. F Boehringer, Mannheim) The tests were carried out in 1 ml microcuvettes and changes in absorbance representing oxidation of NADH to NAD were measured at 3 nm in a Beckmann DUr spectrophotometer by using a tungsten light source

The differences in nucleotide concentration between PRP and PPP have been assumed to indicate the content of ATP, ADP and AMP in the platelets to be tested and have been expressed in mg of nucleotides/10^{11} platelets.

Case Report

G G., a 15 year-old boy was admitted to our department in July 1969 for large hemarthrosis in the right knee and excessive bleeding by minor injuries. He was the first son of 2 first cousins. The patient's parents and the 4 sisters were healthy and have never abnormally bled.

His past medical story disclosed frequent episodes of bleeding from oral cavity in the first years of life and, when he was 6 years old, a profuse hemorrhage after an accidental wound of the scalp, for which he needed several blood transfusions in another hospital. At the admission, there was a large hemarthrosis in the right knee with partial ankylosis and marked muscular hypothrophy of the right leg and thigh. Radiological survey of the right knee showed a decreased density of the bones with multiple areas of rarefication and scattered hyperopacity of periarticular tissues.

Urine analysis, BUN and fasting blood sugar were normal, hemoglobin 12.9 g%, erythrocytes 4,300,000/mm^3, hematocrit 40%, leukocytes 11,200 mm^3 with a normal differential count. Coagulation and hemostasis researches are listed in table I.

AHF-activity, bleeding time and platelet aggregation after human plasma transfusion. The patient was transfused with 400 ml of normal human plasma (8 ml/kg). The results of the variations of AHF activity, bleeding time and platelet aggregation in the days following this transfusion are listed in table II. The AHF-activity raised from 23 to 98% after 6 h and remained elevated (98–93%) up to

Table II. Patient's AHF-activity, bleeding time and platelet aggregation after a normal human plasma transfusion

Normal human plasma AHF-activity %	Patient's plasma AHF-activity % before infusion	after infusion 6 h	12 h	24 h	48 h	72 h
100	23	98	98	93	75	2
Bleeding time, min	>20	right 9 left 13	>20	>20	>20	>20
Platelet aggregation by ADP 10^{-4} M, %	14	42	8	7	6	7

Table III. Normal and patient's platelet nucleotides (mg × 10^{11} platelets)

	Normal platelets				Patient's platelets			
	ATP	ADP	AMP	ATP/ADP	ATP	ADP	AMP	ATP/ADP
1	4.82	1.92	0.31	2.5	5.92	0.61	0.03	9.7
2	4.17	3.40	2.55	1.2	5.26	0.50	0.05	10.4[1]
3	2.92	0.92	1.38	3.1				
4	4.95	2.23	1.44	2.2				
5	5.45	1.47	1.02	3.7				

[1] Patient's platelet nucleotide determinations repeated 10 months after the first admission.

Table IV. Variations of patient's platelet nucleotides after 2 normal human plasma transfusions

Time from the end of the transfusions, h	Platelet nucleotides (mg × 10^{11} platelets)					
	ATP		ADP		AMP	
	1st	2nd[1]	1st	2nd[1]	1st	2nd[1]
0	5.92	5.26	0.61	0.50	0.03	0.05
2	4.66	5.04	1.68	1.79	0.65	0.77
6	10.40	9.42	1.35	1.49	0.67	0.64

[1] The second determinations have been carried out 10 months after the first admission.

24 h, then decreased to 75% after 48 h and to 2% after 72 h from the end of the transfusion. The bleeding time was partially corrected only after 6 h, with values of 9 min at the right ear and 13 min at the left one. In the following observations the hemorrhage lasted more than 20 min and was always stopped with a plug embedded with a thrombin solution. The platelet aggregation by ADP raised from the initial value of 14 to 42% after 6 h from the end of the transfusion. In the subsequent observations the aggregation was always pathological, ranging between 6 and 8%.

Normal and patient's platelet nucleotides. The results of this study are listed in table III. As it may be seen, there has been a high variability in the normal platelet nucleotide content, with an ATP/ADP ratio ranging between 1.2 and 3.7. In 2 experiments the patient's platelet ATP has resulted normal, falling in the ranges of our healthy subjects (5.92 and 5.26 mg × 10^{11} platelets), whereas ADP and AMP were very lowered, strongly below our controls' values (ADP 0.61 and 0.50 mg × 10^{11} platelets, AMP 0.03 and 0.05 mg × 10^{11} platelets). Patient's platelet ATP/ADP ratio has been 9.7 in the first and 10.4 in the second experiment. The

Table V Coagulation and hemostasis tests of 6 family members

Siblings	Clotting time		Bleeding time		Tourniquet test	Platelet count	Clot retraction	Plasma thromboplastin generation test	
	min	sec	min	sec				min	sec
Father	5		2		+ − − −	210 000	+ + + +	8	10
Mother	6	30	1	30	+ − − −	250 000	+ + + +	8	10
1st sister	4	30	3		− − − −	256 000	+ + + +	8	10
2nd sister	5	30	1	30	+ + − −	180 000	+ + + +	8	9
3rd sister	4	50	2	15	− − − −	230 000	+ + + +	8	12
4th sister	3	30	2		+ − − −	261 000	+ + + +	8	10

patient s platelet nucleotide variations after 2 and 6 h from the end of 2 normal plasma transfusions are reported in table IV and may be summarized as follows platelet ATP after 2 h was substantially unchanged (4.66 and 5.04 mg × 10^{11} platelets) whereas ADP and AMP were both strongly increased (ADP 1.68 and 1.79 mg × 10^{11} platelets AMP 0.65 and 0.77 mg × 10^{11} platelets), platelet ATP after 6 h was very increased (10.4 and 9.42 mg × 10^{11} platelets), while ADP and AMP did not substantially differ from the levels of 2 h (ADP· 1.35 and 1.49 mg × 10^{11} platelets AMP 0.67 and 0.64 mg × 10^{11} platelets)

Studies on coagulation hemostasis on 6 family members Both parents and 4 sisters of our patient have been studied. The results are listed in table V All members, studied with clotting and bleeding time, tourniquet test, platelet count, clot retraction and plasma thromboplastin generation test, have given normal results, except for a slight positivity of the tourniquet test in 4 subjects. Unfortunately, it has not been possible to examine the platelet nucleotide content of these family members.

Discussion

There are no doubts that our patient is a case of von Willebrand s disease the AHF defect and the prolonged bleeding time have been the main traits of his hemorrhagic diathesis. Our diagnosis is strengthened by the behavior of the AHF activity that, after the normal human plasma transfusion, has risen to a level much above the predictable concentration calculated from the baseline AHF level and the volume of the transfused plasma. Indeed, according to the formula [38]

$$\text{Predictable plasma factor VIII concentration} = \frac{\begin{pmatrix}\text{before infusion}\\ \text{plasma factor}\\ \text{VIII concentration}\end{pmatrix}\begin{pmatrix}\text{initial}\\ \text{plasma}\\ \text{volume}\end{pmatrix} + \begin{pmatrix}\text{transfused plasma}\\ \text{factor VIII con-}\\ \text{centration}\end{pmatrix}\begin{pmatrix}\text{transfused}\\ \text{SED plasma}\\ \text{volume}\end{pmatrix}}{\text{patient's plasma volume} + \text{transfused plasma volume}}$$

The predictable patient's AHF concentration after transfusion is 36%, with an initial plasma volume of 40 ml/kg [38], a body weight of 50 and calculating the other values from table II The observed concentration of AHF after the transfusion has been 98%, much above the predictable value, according to the theoretic calculation [38]

The difference between the observed and the predictable AHF concentration represents the paradoxical increase of AHF concentration after a normal plasma transfusion This phenomenon, peculiar of von Willebrand's disease, is probably due to an active synthesis of factor VIII by means of one or more unknown transfused factors, present in normal and hemophilic plasma and lacking in these patients' blood

An interesting problem that arises from the study of our case is the *nonfamiliarity of this disease, as both the parents and the 4 sisters have* not shown any alteration of the hemostatic process However, it is not possible to rule out in our patient an autosomal dominant inherited disease with low expressivity in the heterozygous family members, since we have not had the opportunity to examine in these 6 apparently healthy relatives the platelet adhesiveness, that, according to STRAUSS and BLOOM [10], is the only hemostatic defect in some heterozygous carriers of von Willebrand's disease with normal AHF activity and bleeding time From a biochemical standpoint, our patient differs from those studied by CAEN and COUSIN [28 29] for the normal platelet ATP and the strong diminution of both ADP and AMP It is interesting to observe that the platelet nucleotide alterations found by us are similar to those described by these authors in their thrombasthenic patients without glycolytic anomalies, in whom ATP is normal and ADP is strongly lowered, though in our patient the clinical picture has been that of von Willebrand's disease

The temporary partial correction of the bleeding time in addition to the normalization of the platelet aggregating function mediated by exogenous ADP, obtained after the normal plasma inoculation, could be due to one or more unknown transfused factors acting on the hemostatic process through the correction of the biochemical platelet nucleotide

alterations From a clinical standpoint, our observation confirms the data of other authors [7, 18, 21, 24] who have observed the correcting properties of normal plasma *in vivo* and *in vitro* on the defective platelet adhesiveness in some cases of von Willebrand's disease

The correction of platelet aggregation induced by exogenous ADP 6 h after the transfusion has coincided with platelet ATP above the normal values and with an increase of both ADP and AMP from very low levels to normal concentrations

Our data suggest the hypothesis that in our case a normal plasma transfused factor(s) has enhanced with an unknown mechanism a *de novo* synthesis of platelet ADP This newly synthetized nucleotide could have increased subsequently the production of ATP and AMP, probably through the action of a platelet adenylatekinase [39] The so-reached high platelet nucleotide concentration could have been the source of energy for a normal transient platelet aggregation mediated by exogenous ADP This active nucleotide synthesis mediated by the transfused plasma, could have been able to correct, in part, through a normal platelet aggregation, the prolonged bleeding time of our patient.

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Varia

Seminar in Haematology, University of Ibadan

An International Advanced Seminar in Haematology will be held from 4–18 August, 1972, at the Department of Haematology and WHO Regional Reference Center for Glucose 6-Phosphate Dehydrogenase in the Faculty of Medicine, University of Ibadan, Nigeria The general aim of the course is threefold (1) to make available to haematologists and other interested physicians in Africa very up-to-date views of the development of haematology and its public health implications, (2) to make haematologists from outside Africa more aware of clinical material and current work being undertaken here, and (3) to create basis for future advanced research in Africa, including cooperative projects with countries elsewhere

The seminar will include formal lectures, reports on current research, clinical presentations, panel discussions and informal group work During the seminar, a 2-day International Haematology Conference, 7–8 August, will also take place for the exchange of information on haematological research in Africa

Further information can be obtained from the Organizing Committee, Advanced Seminar in Haematology, Department of Haematology, University College Hospital, *Ibadan* (Nigeria)

Jean Julliard Prize

The 4th Jean Julliard Prize, which was established by the International Society of Blood Transfusion in memory of its first Secretary General will be awarded during the 12th International Congress of Blood Transfusion to be held in Washington, D C. (USA), from August 27 to September 2, 1972 The Prize is reserved for investigators under 40 years of age in recognition of recently completed scientific work related to blood transfusion The value of the prize is 3,000 Swiss francs

In order to qualify, candidates must forward 4 copies of an unpublished manuscript or a recently published paper to the Secretary General, Prof J P Soulier, 6, rue Alexandre-Cabanel, *Paris 15e* (France)

The Jury for selecting the recipient of the Julliard Prize was designated during the last meeting of the Executive Committee of the International Society of Blood Transfusion in Washington on May 9 and 10 1971, and consists of the following members Dr T J Greenwalt (USA), President of the ISBT, Prof J P Soulier (France), Secretary General of the ISBT, Dr F H Allen (USA), Dr H R Nevanlinna (Finland)

Acta Haemat 47 193–199 (1972)

Membrane Abnormalities in Polyagglutinable Erythrocytes[1]

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Abstract Polyagglutinable erythrocytes cause difficulty and delay in blood grouping and crossmatching Abnormality of the erythrocyte membrane is the fundamental cause of some types of polyagglutinability The altered chemical structure of the red cell membrane in various forms of polyagglutination is described with particular reference to the application of seed agglutinins in the classification of erythrocyte polyagglutinability T, Tn and Cad polyagglutination receive special attention and the acquired B state is discussed The haematological features of Tn polyagglutination are also described

Key Words
Blood groups
Erythrocyte agglutinins
Erythrocyte antigens
Erythrocyte membrane
Polyagglutination

Polyagglutinable erythrocytes cause difficulty and delay in blood grouping and crossmatching Erythrocyte polyagglutinability may be due (table I) to microbial or nonmicrobial causes

Microbial Polyagglutination

Passive bacteriogenic polyagglutination Bacteriogenic polyagglutination occurs when bacteria or their products are adherent to the erythrocyte surface so that the erythrocytes are agglutinated by sera which contain specific antibacterial antibody [9] In many examples there is direct agglutination of the erythrocytes, in others, erythrocytes are not directly agglutinated but give positive antiglobulin tests, which demonstrate that antibacterial antibody has combined with bacteria adherent to the ery

[1] Paper read at the First Meeting of the European Division of the International Society of Haematology Milan, 10th Sept., 1971

Table I Polyagglutination

	Microbial
1	Adsorption of bacteria or bacterial products Agglutination by sera containing specific antibacterial antibody
2	Removal of sialic acid by bacterial or viral neuraminidase T polyagglutination
3	? Conversion of N-acetylgalactosamine to galactose by a bacterial deacetylase acquired B
	Nonmicrobial
1	Obscure pathogenesis Tn polyagglutination
2	Inherited Cad polyagglutination

throcyte [21] Bacteriogenic polyagglutination is a passive or secondary form of polyagglutination in which there is no intrinsic change in the structure of the erythrocyte membrane

Some forms of drug induced haemagglutination have a similar origin and must therefore be considered in the elucidation of polyagglutination For example, penicillin is readily adsorbed to erythrocytes which are then agglutinable by any serum which contains anti-penicillin antibody

T-polyagglutination This form of polyagglutination is associated with a change in erythrocyte membrane structure It is due to the action of bacterial or viral neuraminidase on the red cell membrane [12] Neuraminidase splits off N-acylneuraminic (sialic) acid from the red cell surface and exposes a latent receptor, the T-antigen of FRIEDENREICH [10] Since anti-T is present in most adult mammalian sera, T-transformed erythrocytes are polyagglutinable In T-transformation removal of sialic acid by neuraminidase exposes a β-galactosyl residue which is the chief structural determinant of T-specificity [20]

T-polyagglutination usually occurs as the result of *in vitro* contamination of blood specimens, some examples of *in vivo* T-polyagglutination have also been described

It is now possible quickly to identify T-transformed erythrocytes They are strongly agglutinated by extract of peanuts *(Arachis hypogaea)* which contains a powerful anti-T agglutinin [4] Furthermore, T-transformed erythrocytes are not aggregated by polybrene, a substance which strongly aggregates normal erythrocytes [14] In fact, loss of as little as 5–7% of sialic acid renders erythrocytes unclumpable by polybrene [13]

Acquired B In the acquired B condition, erythrocytes become agglutinable by many, but not all, group O or A sera Acquired B may there-

fore be considered to be a form of polyagglutination. It occurs only in A_1- and never in O-persons It is thought that a bacterial deacetylase is responsible [11] This enzyme converts α-N-acetylgalactosamine, the structural determinant of A-specificity to α-galactose, the structural determinant of B-specificity

Nonmicrobial Polyagglutination

Tn polyagglutination Tn-polyagglutination is an acquired *in vivo* condition which is characterised by a 'mixed field' reaction due to the fact that 2 erythrocyte populations are present, one of which is agglutinated by anti-Tn present in most human adult sera The 2 populations can be separated by electropheresis, one population is appreciably deficient in sialic acid [17] Although Tn-cells are deficient in sialic acid, they are not agglutinated by peanut extract. Polybrene gives a 'mixed-field reaction with Tn-cells which is quite the opposite of that produced by human sera. Polybrene fails to aggregate agglutinable erythrocytes and strongly aggregates nonagglutinable erythrocytes

Tn erythrocytes are strongly agglutinated irrespective of blood group by extracts of the seeds of *Dolichos biflorus* [15], a well known source of anti A agglutinins [1] The reaction is specifically inhibited by N-acetylgalactosamine This suggests that N-acetylgalactosamine is the chief structural determinant of Tn-specificity *Dolichos biflorus* extract does not agglutinate T-transformed cells [3]

Although both T- and Tn polyagglutinable erythrocytes are deficient in sialic acid, it is clear from the reactions of the peanut and *Dolichos biflorus* agglutinins that whereas in T-transformation sialic acid is split off from β galactosyl groups, in Tn-polyagglutination the absence of sialic acid leaves N acetylgalactosamine residues exposed

Tn-cells are strongly agglutinated by extracts of *Bauhinia variegata* or *Bauhinia purpurea* seeds, which contain anti N agglutinins, and those of *Moluccella laevis* seeds which contain crossreacting anti-(A+N) agglutinins [6] These reactions are also specifically inhibited by N acetylgalactosamine [7]

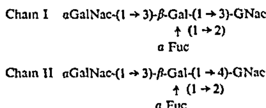
There is increasing evidence that the Tn-condition constitutes a definite haematological syndrome characterised by 'mixed field' erythrocyte polyagglutinability, leucopaenia and thrombocytopaenia [7] Leucocytes and platelets, however, are not polyagglutinable The involvement of

erythrocytes, leucocytes and platelets suggests that the site of action of the factor (or factors) which produce the Tn-change operates in the bone-marrow rather than in the blood stream. Haemolytic anaemia has been reported in some patients with Tn-polyagglutinable erythrocytes. This is not surprising because a number of structural defects of the erythrocyte membrane are associated with haemolytic anaemia, e.g. the Rh_{null} state.

Cad-polyagglutination. Cad is an inherited dominant character. Cad erythrocytes are polyagglutinable because anti-Cad is present in most human adult sera. Cad cells are very strongly agglutinated, irrespective of blood group, by *Dolichos biflorus* seed extract, which contains separable anti-A_1 and anti-Cad agglutinins [8]. Both agglutinins are specifically inhibited by N-acetylgalactosamine. The sialic acid content of Cad erythrocytes is normal.

Differentiation of T-, Tn- and Cad-polyagglutination. The 3 kinds of polyagglutination may be distinguished from one another in accordance with the scheme shown in table II.

N-acetylgalactosamine. In human erythrocytes, N-acetylgalactosamine is the chief structural determinant of 3 different receptors: A, Tn and Cad. The blood group A determinant has the structure [16]:



The chemically similar Forssman antigen probably has the structure [18]: α GalNac (13) β gal (1→4) GNac, or α-GalNac (1→3) β gal (1→3) GNac, and differs from human A only in having no α-fucosyl residue linked (1→2) to the subterminal galactose.

Despite the chemical similarity of human A and the Forssman antigen, there is evidence that *Dolichos biflorus* activity is not anti-Forssman [2]. It may therefore be assumed that neither the Tn nor Cad determinants are absolutely identical with the Forssman receptor. In this connection it might be mentioned that rabbit anti-sheep erythrocyte serum (anti-Forssman) agglutinates A but not Cad erythrocytes.

The precise structures of the Tn- and Cad receptors are yet to be determined.

Table II Comparison of T Tn and Cad polyagglutination

Origin	T acquired (microbial neuraminidase)	Tn acquired (unknown cause)	Cad inherited
Agglutination by *Arachis hypogaea* (peanut)	+	–	–
Agglutination by *Dolichos biflorus*	–	+	+
Aggregation by polybrene	–	mixed field	+
Effect of papain on receptor	no discernable effect	destroyed	enhanced
Chemical basis of specificity	β-galactose	N acetylgalactosamine	N acetylgalactosamine

Cad and Sid Evidence has recently been obtained which indicates that Cad is an abnormally strong form of the erythrocyte antigen Sid or Sd^a, so strong that the erythrocytes are polyagglutinable [19] This means that although the majority of persons are Sd(a+) anti Cad (anti Sd^a) is present in the majority of sera. It must therefore be assumed that if Cad is indeed Sd^a, the anti Sd^a present in most sera is incapable of agglutinating Sd(a+) cells Such a situation is well known in blood group serology: A_1 persons have weak H on the red cells and may have anti H acting only at low temperatures, in the serum. Inhibition studies using materials rich in Sd^a substance, which will be described in detail elsewhere show that Sd^a substance strongly inhibits the anti-Cad of *Dolichos biflorus* These observations support the view that Cad is Sd^a

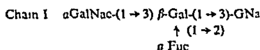
Other membrane changes in T-, Tn and Cad polyagglutination In T transformation the M and N antigens, as measured by human or animal anti M and anti N are destroyed and the action of seed anti M and anti N is enhanced This is because M and N specificity is determined partly by sialic acid with which human and animal anti M and anti N react, and partly by another carbohydrate, probably a galactosyl or substituted galactosyl residue, with which seed anti M and anti N react. Some Tn erythrocytes have enhanced H, some enhanced D and c, and

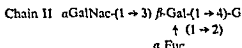
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Acta Haemat 47 200–209 (1972)

Iron Metabolism and Red Cell Survival in Uremic Patients

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Key Words
Erythrocyte survival
Iron metabolism
Renal anemia
Uremia

Abstract In 12 patients with chronic renal failure iron utilization and erythropoiesis have been studied by administration of ^{59}Fe and estimation of red cell survival time with ^{51}Cr Quantitative studies of the clearance of ^{59}Fe from the plasma and its incorporation into the circulating red cell show that erythropoiesis is in certain cases effective and in other cases ineffective These findings are confirmed by body surface measurements with radioactive iron Studies with radioactive chromium also show in certain cases a normal and in others a reduced survival time of the circulating red cells Thus the cause of anemia in uremic patients seems to be due either to a diminished red cell production hemolysis, or a combination of both

Chronic renal failure is usually accompanied by anemia of varying degree This type of anemia, though known since Bright described nephritis, has been investigated thoroughly only in the last 15 years It has been shown [2] that the anemia is associated with an absent or reduced plasma erythropoietin activity when compared with anemia due to other causes This considered as the main reason of the anemia. On the other hand slight hemolysis, which in rare cases is severe has been found to occur in renal failure Subsequently, a shortening of the red cell life span has been shown to be a common finding in renal anemia by different studies with radioactive chromium (^{51}Cr) [6 16] The etiology of this hemolysis, as well as that of reduced hemopoiesis, are still not clarified

The purpose of this work was to simultaneously study erythropoiesis by radioactive iron (^{59}Fe) and the red cell survival time by ^{51}Cr in 12 patients suffering from various degrees of renal failure

Subjects and Methods

Table I shows the sex, age, diagnosis and laboratory findings of these patients. The investigation of each patient was started a few days after hospitalization, when the electrolyte imbalance was corrected and water equilibrium restored. Digitalis was given in the presence of congestive heart failure. Those patients who had severe anemia were subjected first to red cell transfusion, while during the period of the study no transfusion was given. Iron or any other drugs were also not administered to the patients. In 10 patients, a combined study of the red cell survival time and the iron metabolism was performed, while in 2 patients, only the red cell survival time was measured.

The method used was that described by Malamos *et al.* [11]. A sample of the patient's own plasma was incubated *in vitro* with 10 μCi ^{59}Fe, and the mixture injected intravenously. Blood samples were withdrawn during the first hour at 10-min intervals. The plasma of these samples was separated and assayed for ^{59}Fe (from the plasma, the ^{59}Fe clearance curve was determined). In the following 2 weeks, blood samples were withdrawn every 2 days and assayed for ^{59}Fe (from the red cell ^{59}Fe uptake curve, the percentage uptake of ^{59}Fe in the red cells and the red cell iron turnover were obtained). During the study at frequent intervals, body surface counting measurements were made over the sacrum, liver and spleen rating of the ^{59}Fe turnover by the tissues followed.

Two days after the injection of ^{59}Fe, a sample of the patient's red cells was labelled *in vitro* with 100 μCi ^{51}Cr and reinjected. Blood samples were withdrawn at 1 and 24 h, and every 3 days for 3 weeks, and assayed for ^{51}Cr. From the ^{51}Cr survival curve, the half-survival time ($T^1/_2$) was estimated. At frequent intervals body surface counting measurements were made over the spleen and liver and the importance of these organs as sites of red cell destruction assessed.

Results

Table II shows the radioisotopic findings. The patients under investigation, regardless of the etiology of their renal insufficiency, presented anemia. From 10 examined patients, the number of reticulocytes was normal in 6 and elevated in 4. Serum iron was normal or slightly decreased in 7, markedly elevated in 2, and decreased in 1.

The patients who were investigated can be divided into 3 groups, according to the percentage of ^{59}Fe incorporation in their red cells (fig. 1 and 2).

Group I (cases 1, 2, 3, 7, 10). In this group, the plasma iron half-clearance time and the incorporation of ^{59}Fe into the circulating red cells were normal (fig. 3). The plasma iron turnover and the red cell iron turnover were within normal limits or slightly deviated (0.32–0.84

Table I

Case No	Sex	Age	Diagnosis	Blood or serum							
				urea, mg%	creatinine, mg%	uric acid, mg%	hemoglobin, g%	hematocrit, %	reticulocytes, %	serum iron, μg/100 ml	mean 24 h urinary output
1	M	45	gouty nephropathy	87	4.90	8.3	10.6	33	1.0	116	1,100
2	M	50	gouty nephropathy	60	1.93	8.0	13.2	42		115	1,000
3	M	62	chronic pyelonephritis	140	5.37	9.1	8.8	29	5.0	98	1,700
4	M	67	chronic glomerulonephritis	126	3.13	4.6	9.1	30	1.2	76	1,500
5	M	45	chronic glomerulonephritis	75			7.9	26			1,050
6	M	72	chronic glomerulonephritis	110	3.37	7.8	8.8	31	2.1	22	900
7	F	53	chronic pyelonephritis	117	6.00	9.6	8.7	25	1.4	78	1,000
8	M	70	chronic pyelonephritis	76			10.8	33	1.3		
9	F	54	chronic pyelonephritis	92	2.80	4.8	10.0	32.5	0.8	69	1,300
10	F	50	chronic pyelonephritis	77	6.70	10.3	11.1	35.9	0.8	89	900
11	M	55	chronic glomerulonephritis	109	9.60	10.6	7.7	23.5	2.4	153	1,100
12	M	44	polycystic kidneys	240	4.10	10.2	12.0	41	7.4	143	1,400

Table II

Case No	Diagnosis	Plasma ^{59}Fe half-clearance, min	Red cell ^{59}Fe uptake %	Plasma iron turnover rate mg/kg/day	Red cell iron turnover rate mg/kg day
1	gouthy nephropathy	105	94	0 7	0 66
2	gouthy nephropathy	61	90	0 83	0 79
3	chronic pyelonephritis	67	95	0 84	0 80
4	chronic glomerulonephritis	107	70	0 32	0 21
5	chronic glomerulonephritis	65	75		
6	chronic glomerulonephritis	80	80	0 44	0 36
7	chronic pyelonephritis	80	100	0 51	0 51
8	chronic pyelonephritis				
9	chronic pyelonephritis	72	80	0 62	0 50
10	chronic pyelonephritis	116	88	0 32	0 29
11	chronic glomerulonephritis				
12	polycystic kidneys	85	63	0 64	0 39
Normal values		60–120	85–100	0 46–0 75	0 43–0 72

and 0 29–0 80 mg/kg daily, respectively) Surface measurements over the sacrum (bone marrow) revealed an effective erythropoiesis, with the exception of case 3, in which the counting rate fell slowly The last finding was in good agreement with the ^{59}Fe incorporation curve, which rose slowly without reaching the maximum until the 14th day In 2 patients (cases 1 and 7), a secondary rise in the spleen and liver counting rates was observed The survival of ^{51}Cr-labelled red cells in the circulation of these 2 patients was reduced, whilst surface counting studies revealed a moderate accumulation of ^{51}Cr in the spleen and in the liver The remaining 3 patients had a normal, or almost normal, survival of ^{51}Cr labelled red cells

Group 2 (cases 6 and 9) In this group, the plasma iron half-clearance time was normal, but the incoporation of ^{59}Fe into the circulating red cells was reduced. The plasma iron turnover and the red cell iron turnover were almost within normal limits. Surface measurements over the sacrum are in good correlation with the above findings, showing a delay in erythropoiesis and restoration to its extrapolated value at zero time

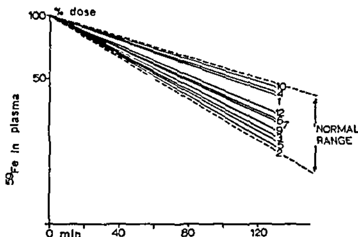


Fig 1 Plasma ^{59}Fe half clearance time

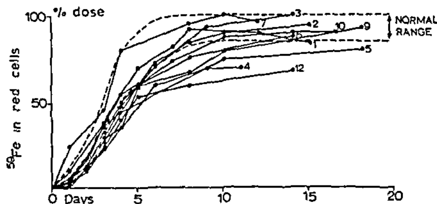


Fig 2 Incorporation of ^{59}Fe into the red cells

The survival of the ^{51}Cr-labelled red cells in the circulation was normal in one patient and reduced in the other Surface counting studies in the latter revealed a deposition of ^{51}Cr in the spleen and liver

Group 3 (cases 4, 5, 12) In these patients, the ^{59}Fe half-clearance time was normal, but the red cell ^{59}Fe uptake curve rose more slowly than in normal subjects, reaching a maximum value which was lower than the normal (fig 4) The plasma iron turnover fluctuated between normal values and a little below normal, whereas the red cell iron turnover was found to be below normal Surface measurements over the sacrum

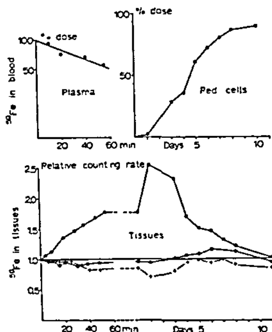


Fig 3 Studies with ^{59}Fe in a uremic patient with normal erythropoiesis (case 2) ● Bone marrow, ○ spleen, × liver

showed erythropoiesis to be effective in one patient, delayed in the second and ineffective in the last. The survival of ^{51}Cr labelled red cells in the circulation was normal in one patient and reduced in the other two Surface counting studies revealed ^{51}Cr deposition in the liver of one of the latter two patients

Comments

Iron Metabolism

The present study, by the use of ^{59}Fe, showed that erythropoiesis was, in some cases, effective, in some, delayed, and in only one ineffective (fig 2) Our findings agree with the results of ESCHBACH *et al* [5] and KURTIDES *et al* [8] In the groups of patients with a normal or slightly reduced erythropoiesis, the plasma iron turnover, representing whole erythropoiesis, and the red cell iron turnover, representing effective erythropoiesis, were within normal limits, with slight fluc-

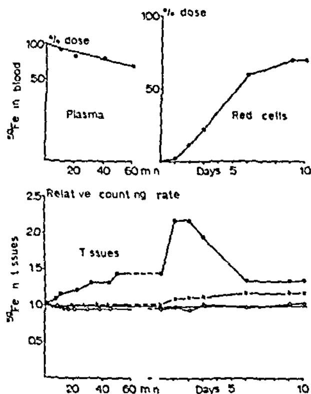


Fig. 4. Studies with ^{59}Fe in a uremic patient with ineffective erythropoiesis (case 4). For symbols see figure 3.

tuations. In the group of patients with reduced erythropoiesis, the plasma iron turnover was almost within normal limits, but the red cell iron turnover was below normal. This has been attributed to the fact that the plasma iron turnover represents whole, and not effective, erythropoiesis or attributed to transfer of iron to tissues other than bone marrow [1, 14].

It is questionable whether the observed abnormality of the iron metabolism is due to a disturbance of erythropoiesis. It is considered that the main cause is the reduction of erythropoietin production by the kidneys, observed in azotemic conditions [7].

Red Cell Survival

Patients with reduced survival of the ^{51}Cr labelled red cell were found in all 3 groups (fig. 5). It seems strange that in 2 cases with a nor-

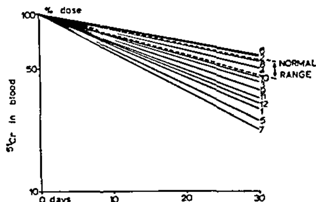


Fig 5 ^{51}Cr half-survival time

mal incorporation of ^{59}Fe, the red cell survival was reduced The red cell ^{59}Fe uptake curve, after reaching the maximum value, fell in one patient after the 12th, and in the other, after the 15th, day This seems indicative of hemolysis A similar observation was made in a patient studied by MAGID and HILDEN [10] In the literature, there is disagreement on the existence of a reduced red cell survival in uremic subjects Thus, RAGEN *et al* [12] came to the conclusion that reduced red cell survival is, in chronic renal insufficiency, a rare finding, while DESFORGES and DAWSON [4] found it a usual finding This points out the difficulty in the determination of red cell survival time in chronic renal insufficiency Water and electrolyte disturbances cause wide fluctuations of the blood volume and, consequently, influence the radioactivity of the whole blood samples

CHAPLIN and MOLLISON [3] did not find a correlation between the red cell survival time and the urea nitrogen level They observed, however, a higher correlation with the quantity of urinary ouput. The patients of LOGE *et al* [9], with a short red cell survival time, had higher values of nonprotein nitrogen, compared to those with a normal red cell survival time SHAW [15] found that shortening of the red cell survival parallels the rise of blood urea levels DESFORGES and DAWSON [4] have shown by cross experiments that in uremic patients, the survival of red cells transfused to normal subjects is normal, while the survival of red cells of normal subjects transfused to uremic patients is reduced.

It seems, therefore, that extracellular factors of the blood plasma influence the red cell survival in these patients Blood urea does not seem to be included among these factors Other substances whose level parallels that of urea should be implicated These substances are probably micromolecular, and pass the semipermeable membranes, because it has been observed that in uremic patients, following hemodialysis, there is a decrease in hemolysis [15] In chronic renal insufficiency, the excretion of such substances is relatively unaffected until the glomerular filtration rate falls to very low levels [16] Other investigators talk of a chemical change of the red cells which renders them fragile [13] In our patients, no correlation was found between urea blood levels and red cell survival time However, when the quantity of urine is considered, there seems to be some correlation

The anemia in our patients was generally in good agreement with the radioisotopic findings, although an apparent disagreement may sometimes be observed Thus, in the anemic patients with a normal erythropoiesis (cases 1 and 7), the anemia was due to the observed reduced red cell survival time In 2 patients, one with a normal erythropoiesis (case 3) and the other with a slightly reduced erythropoiesis (case 6), the existing severe anemia did not result from the shortened red cell survival time KURTIDES *et al* [8] explain this disagreement They state that whenever the serum iron level is low, as in one of our patients, the specific radioactivity of the serum iron is increased, resulting in a falsely high incorporation of iron in the red cells

It can be concluded that the anemia in our uremic patients is sometimes due to ineffective erythropoiesis, in others, it is due to a shortening of the red cell survival time and often to a combination of both, a fact which permits the hypothesis that the observed anemia results probably from more than one factor

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Acta Haemat. 47 210–216 (1972)

Haemoglobin E-Hereditary Elliptocytosis in Malayan Aborigines[1]

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Abstract A survey was made of 1,384 Malayan aborigines for frequencies of abnormal haemoglobin and hereditary elliptocytosis The subjects were 137 healthy adults (93 jungle police and 44 hospital personnel), 384 patients from the Ulu Gombak Aborigine Hospital, and 863 villagers from northern Pahang and Perak in West Malaysia This led to the finding of 61 cases of haemoglobin E (Hb E)-hereditary elliptocytosis, a new genetic combination, 32 of the cases were studied in more detail In those aborigines who did not have other diseases, the combination of the 2 abnormal genes was not associated with clinical symptoms or significant haematological abnormalities Family studies showed that the genes for Hb E and for hereditary elliptocytosis were inherited independently

Key Words
Elliptocytosis
Haemoglobin E
Haemoglobinopathies
Malaya

High frequencies of haemoglobin E (Hb E), ranging from 8 to 54%, have been found in different ethnic groups of Malayan aborigines by LIE-INJO and CHIN [8] and LIE-INJO and BOLTON [unpublished] Frequencies for hereditary elliptocytosis are also high in these groups, a frequency of more than 12% was reported in one study [7] Therefore, the combination of the genes for Hb E and hereditary elliptocytosis may be expected in these aborigines, several instances were mentioned by

[1] This work was supported by research grant AI 10051 to the University of California International Center for Medical Research and Training and by research grant HE 10486, Dr A FIX was supported by fellowship 5 FOI MH 34135-04, with attached grant MH-11415-01, all from the National Institutes of Health, US Public Health Service



Fig 1 Map of West Malaysia. Places in Pahang and Perak where blood samples were collected are indicated (X). Shaded area is where Senoi aborigines are found [13]

LIE INJO in her earlier study [7]. In this paper, we report on the clinical, haematological, and family studies of this genetic combination.

Material and Methods

The Malayan aborigines are comprised, according to WILLIAMS-HUNT [13], of 3 main ethnic groups: the Negritos, the Senoi, and the Aboriginal Malays, each consisting of many subgroups. Blood was obtained for study of abnormal haemoglobin and hereditary elliptocytosis from 3 groups of subjects (not divided ethnically): (1) 137 unrelated healthy adult Malayan aborigines, mostly Senoi (93 jungle police and 44 hospital personnel) who were under constant medical supervision; (2) 384 patients, mostly adults of various ethnic groups, who had been admitted to the Ulu Gombak Aboriginal Hospital for treatment of various diseases (this hospital serves all aborigines throughout Malaysia); (3) 863 inhabitants of dif

Table I Hb E-hereditary elliptocytosis in Malayan aborigines

Group	Number examined	Hb E	Hereditary elliptocytosis	Hb E hereditary elliptocytosis[1]
Healthy personnel				
Jungle police	93	30 (32.3%) (AE 20, EE 10)	12 (12.9%)	2 (AE)
Hospital personnel	44	15 (34.1%) (AE 15, EE 0)	8 (18.2%)	2 (AE)
Patients	384	100 (26.0%) (AE 88, EE 12)	50 (13.0%)	9 (AE)
Villagers				
From northern Pahang	531	202 (38.0%) (AE 173, EE 29)	111 (20.9%)	42 (38 AE, 4 EE)
From Perak	332	149 (44.9%) (AE 129, EE 20)	16 (6.6%)	6 (AE)
Totals	1,384	496	197	61

[1] Also included in the columbs for Hb E and hereditary elliptocytosis

ferent villages in the States of Perak and Pahang. We obtained venous blood from 465 villagers, only fingerpick blood was obtained from the remaining 398. The 531 villagers in northern Pahang (fig. 1) belong to the Semai subgroup of the Senoi. We tried to obtain blood from all villagers in the area, and succeeded in doing so from 73.4% of the population. The 332 Perak villagers (fig. 1) were also primarily Semai.

Haematological examinations followed standard methods. Paper electrophoresis was used for analysis of abnormal haemoglobins at the start of the study, but this was later supplanted by the starch gel method of SMITHIES [12]. The criterion for diagnosing hereditary elliptocytosis was a finding of more than 50% elliptocytes. In many cases, almost all cells were elliptic, usually the experienced eye can readily distinguish this abnormality from the symptomatic elliptocytosis accompanying secondary anaemias. Estimation of alkali resistant haemoglobin followed the method of SINGER *et al.* [11]. Hb A_2 was quantitated by the method of MARENGO-ROWE [9].

Results

Table I shows the frequencies of Hb E, hereditary elliptocytosis and the presence of both in Malayan aborigines in the groups sampled. A to-

tal of 61 persons carried both traits, we obtained blood from 32 of the persons for haematological studies and these people were clinically examined From the healthy group, 2 jungle policemen and 2 hospital employees were found to have Hb E hereditary elliptocytosis All 4, healthy, clinically normal and without spleen or liver enlargement, were heterozygous for Hb E Their haemoglobin levels ranged from 13 9 to 14 6 g/100 ml, packed cell volume (PCV), from 44 to 51%, RBC, from 5 4 to 5 8 million per mm³, mean corpuscular volume (MCV), from 81 4 to 86 2 μm³, mean corpuscular haemoglobin (MCH), from 25 2 to 26 5 pg, mean corpuscular haemoglobin concentration (MCHC), from 29 6 to 32 6% Leucocyte and platelet counts were within normal limits Most red cells were elliptocytes, differing little from those seen in the hereditary elliptocytosis trait, but looking quite different from those seen in persons with the homozygous Hb E condition or the Hb E trait. The fragility in saline of the red cells was normal or slightly decreased. Alkali resistant haemoglobin was within normal limits

The 9 patients with Hb E hereditary elliptocytosis were all heterozygous for Hb E Their symptoms were apparently due to disorders for which they had been admitted to hospital, anaemia in 4 and leucocytosis in 2 were probably due to diseases other than Hb E hereditary elliptocytosis However, all 9 cases showed that this combination does not lead to abnormalities in the levels of Hb F, which were within normal limits

Of the 42 villagers from northern Pahang who had Hb E hereditary elliptocytosis 4 were homozygous for Hb E, and the rest, heterozygous We were able to study only one of the former, but 15 of the latter All came from remote, extremely malarious areas Physical examination showed that 10 had enlarged spleens and livers, 3 had enlarged spleens only, and 3 were physically normal Many were anaemic, and we felt that clinical and haematological findings for them could not be used to characterize symptoms related to their having Hb E hereditary elliptocytosis Examination of the small amount of blood obtained during the general survey from 3 of the 4 villagers homozygous for Hb E and having elliptocytosis showed that they had a slight increase of Hb F They had slightly more pronounced anisocytosis and poikilocytosis than would be expected with heterozygous Hb E hereditary elliptocytosis, or either trait separately, or homozygous Hb E alone

Three of the 6 adult villagers from Perak with Hb E hereditary elliptocytosis were found to be healthy, without palpable spleens and livers. Their haemoglobin levels ranged from 12 5 to 13 9 g/100 ml, PCV,

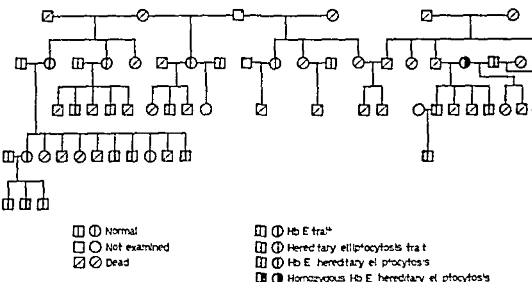


Fig 2 Pedigree of a family with Hb E and hereditary elliptocytosis showi the independent inheritance of the gene for Hb E and that for hereditary elliptoc tosis.

from 38 5 to 49 0%, RBC, from 4 35 to 5 44 million per mm³, MC from 88 5 to 96 9 μm³, MCH, from 25 2 to 28 7 pg, and MCHC, fro 28 3 to 32 9% WBC and platelet counts were normal, and Hb F w within normal limits

Family studies in 44 cases showed that the genes for Hb E and f hereditary elliptocytosis were inherited independently (fig 2)

Discussion

The clinical and haematological findings in the 2 healthy jungle pc licemen and in the 2 hospital employees, all of whom had both Hb and hereditary elliptocytosis, indicate that the genes for Hb E and hered itary elliptocytosis do not interact – at least, not noticeably so The persons with this genetic combination could perform their daily task without impairment, did not have clinical symptoms, and did not hav anaemia or recognizable signs of haemolysis Their haematological find ings were within the normal limits we obtained for a normal contr group of aboriginal villagers In the 9 hospital patients who had bot

Hb E and hereditary elliptocytosis haematological abnormalities were found when they were in the hospital These abnormalities were apparently due to the disease for which they were admitted, not to their being doubly heterozygous for Hb E and hereditary elliptocytosis, when 2 of them were re-examined later, they were found to be healthy, and haematological findings did not show significant changes, although they still had Hb E and hereditary elliptocytosis

Of the villagers with the combination of Hb E and hereditary elliptocytosis, some were heterozygous for Hb E, and others, homozygous In the latter, we found more anisocytosis and poikilocytosis, and more irregularities than in villagers doubly heterozygous for both traits and those with hereditary elliptocytosis only In contrast, in persons homozygous for Hb E without hereditary elliptocytosis, the cells were more regular, and the only common abnormality was the presence of many target cells Unfortunately, iron deficiency which could have led to the more pronounced changes could not be excluded The finding of an increased amount of Hb F in homozygous Hb E hereditary elliptocytosis is not surprising, because homozygous Hb E by itself usually leads to a slight increase of Hb F The clinical finding of an enlarged spleen is also fairly common for homozygous Hb E disease Moreover, northern Pahang is still highly malarious, despite recent regular administration of chloroquine tablets to the inhabitants Although the villagers under study were free of malaria parasites at the time of examination, many had enlarged spleens The gene for Hb E and that for hereditary elliptocytosis are inherited independently, as can be seen in figure 2, giving the pedigree for one of the many families examined

Hereditary elliptocytosis has been described in association with Hb S [14] and with Hb C [4, 14] as not having produced clinical or haematological symptoms Combination with β thalassaemia leads to mild anaemia and haematological symptoms, usually without mutual enhancement [1, 5, 6] but several reports have given evidence of some mutual enhancement [2, 3, 10]

Acknowledgements We wish to thank ONYAH s o ITAM of the Orang Asli Medical Service for his valuable help in tracing and taking blood from patients and their relatives in the jungle and for obtaining repeat blood samples the Royal Malaysian Air Force for providing helicopter service to reach the aboriginal villages and TAN SRI DR MOHAMAD DIN BIN AHMAD, the Director of Medical Services of Malaysia, for permission to publish this article.

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Acta Haemat. 47 217-224 (1972)

Anaemia and Erythrocyte Transketolase Activity

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Abstract Erythrocyte transketolase activity is raised in megaloblastic anaemia due to vitamin B_{12} deficiency and inversely related to haemoglobin concentration It si usually normal in cases of megaloblastic anaemia due to folate deficiency though sufficient exceptions occur to invalidate the use of red cell transketolase assay in differentiation of vitamin B_{12} from folate deficiency

Key Words
Erythrocyte transketolase
Folate deficiency
Megaloblastic anaemia
Thiamine deficiency
Vitamin B_{12} deficiency

There is no evidence that altered thiamine metabolism is a factor in the distu ance of pyruvate metabolism that occurs in vitamin B_{12} deficiency, nor that th mine deficiency is involved in the commonly associated neuropsychiatric disorder

Erythrocyte transketolase activity is normal in untreated iron deficiency an mia, aplastic anaemia polycythaemia, chronic myeloid and lymphatic leukaemia, ythroleukaemia and non-anaemic patients with glucose-6-phosphate deficiency creased red cell transketolase activity is seen in iron deficiency anaemia respond to treatment, hereditary spherocytosis and occasionally in patients with an auto mune haemolytic anaemia or acute leukaemia.

Erythrocyte transketolase activity is raised in cases of macrocytic a nemia due to vitamin B_{12} deficiency but normal when it is due to fol deficiency [17, 24] As neither vitamin B_{12} nor folate are known to aff the pentose monophosphate pathway of glucose metabolism, there is no re dily apparent explanation for this observation, which deserves further stu

Patients with pernicious anaemia and neuropathy were shown Hornabrook and Marks [12] to have raised blood pyruvate levels wh were restored to normal by large doses of thiamine This led to suggestion that thiamine deficiency might be responsible for the abn mality of pyruvate metabolism observed and contributed towards the n ropsychiatric manifestations associated with vitamin B_{12} deficiency

Measurement of red cell transketolase activity and experience of thiamine pyrophosphate effect (TPPE) – defined as the percentage

crease in red cell transketolase activity produced by addition of thiamine pyrophosphate to the haemolysate – has proved useful, both clinically and experimentally, as an index of thiamine deficiency [7, 9, 10, 26] We decided therefore to measure TPPE together with fasting blood pyruvate level in a group of patients with vitamin B_{12} deficiency in an attempt to define the relationship, if any, between vitamin B_{12} and thiamine deficiency and determine their role in the aetiology of neuropsychiatric symptoms

Erythrocyte transketolase activity and TPPE was also studied in patients with various haematological diseases

Materials and Methods

Thirty seven patients with pernicious anaemia with and without neuropsychiatric involvement 17 with folate deficiency, 7 with iron deficiency anaemia 12 with haemolytic anaemia of diverse types and 8 with leukaemia were studied Routine haematological studies [6] serum [23] and red cell folate [11] serum vitamin B_{12} [20] and vitamin B_{12} absorption studies [5] were performed where appropriate Red cell transketolase activity total transketolase activity and TPPE [24] blood pyruvate and α ketoglutarate [19] were measured by methods previously described

Results

Mean red cell transketolase activity (93 7 IU/l ± 30 5 SD) was significantly greater ($p<0\ 01$) in 37 cases of megaloblastic anaemia due to vitamin B_{12} deficiency compared with a series of age matched controls (mean 59 7 IU/l ± 12 7 SD) Red cell transketolase activity (mean 52 5 IU/l ± 22 0 SD) was generally within normal range in cases of macrocytosis due to folate deficiency though exceptionally both high and low values were seen (fig 3)

The negative correlation previously observed [24] between haemoglobin concentration and red cell transketolase activity in macrocytosis due to vitamin B_{12} deficiency was confirmed ($r = 0\ 3$ $p<0\ 02$) The correlation was better when total transketolase activity rather than transketolase activity alone was considered ($r = 0\ 47$, $p<0\ 001$) (fig 1) There was no significant relationship between haemoglobin concentration and red cell transketolase activity in patients with folate deficiency

In patients with pernicious anaemia, erythrocyte transketolase activity had usually returned to within the normal range after 3 to 5 weeks treatment with vitamin B_{12} though sometimes it continued to fall for as long as 9 weeks before stabilising (fig 2)

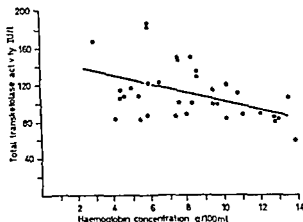


Fig 1 Correlation between the total erythrocyte transketolase activity and haemoglobin concentration in 37 patients with pernicious anaemia. $r = -0.47$, $p < 0.001$ $y = -4.78X + 149.34$

The TPPE was raised in only 2 out of the 37 patients with pernicious anaemia but was elevated in 6 out of 17 patients with nutritional folate deficiency These results support our previous contention [24] that thiamine deficiency is commoner in folate deficient subjects than in patients with pernicious anaemia, in whom it is rare

The result of simultaneous measurement of TPPE and fasting blood pyruvate levels in 8 patients with pernicious anaemia and 4 patients with nutritional folate deficiency are shown in table I In this small group of subjects, there was no discernible relationship between fasting pyruvate levels (which were often raised in both vitamin B_{12} and folate deficiency) and the haemoglobin concentration, transketolase activity, TPPE, serum and RBC folate activity

α-Ketoglutarate levels were depressed to below the normal range in 6 of the 8 subjects with pernicious anaemia studied This is in agreement with observations in B_{12} subjects made by BUCKLE [3] and at variance with those obtained in patients suffering from thiamine deficiency [4]

There was no correlation between TPPE and the presence or absence of neuropsychiatric disorders either in patients with pernicious anaemia or folate deficiency

Red cell transketolase activity was within the normal range in patients with untreated iron deficiency, aplastic anaemia, polycythaemia, chronic

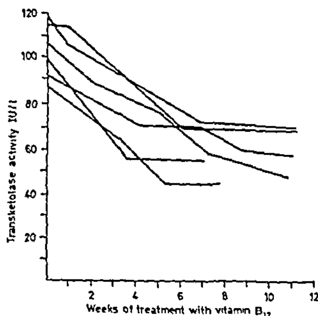


Fig 2 Change in erythrocyte transketolase activity observed during treatment with vitamin B_{12} in 6 patients with pernicious anaemia

myeloid and lymphatic leukaemia, erythroleukaemia, pregnancy and glucose-6-phosphate deficiency in the absence of haemolysis However, raised values were often seen in patients with iron deficiency anaemia who were responding to treatment Red cell transketolase levels were consistently raised in patients with hereditary spherocytosis and occasionally so in patients with autoimmune haemolytic anaemia These data are summarized in figure 3

Discussion

The raised concentration of many of the intracellular red cell enzymes found in megaloblastosis may represent an adaptive process whereby intracellular ATP concentration is maintained If this is so the increased erythrocyte transketolase activity observed in pernicious anaemia would be merely a specific example of a general phenomenon

Red cell transketolase activity is dependent on at least 2 variables (a) the intrinsic concentration of the apoenzyme (qua protein), (b) availability of its co-enzyme thiamine pyrophosphate Thiamine appears to be necessary for the transketolase apoenzyme to demonstrate enzyme activity, even minor degrees of thiamine deficiency perceptibility decreases red cell transketolase activity [2, 25], which can be restored, at least in part, by

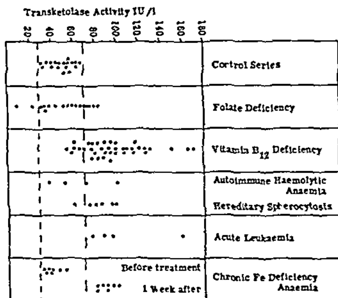


Fig 3 Red cell transketolase activity in various haematological conditions.

addition of thiamine pyrophosphate to haemolysate *in vitro* This has been utilized clinically as a test of B_1 deficiency

Multiple vitamin deficiencies have been described in association with the vitamin B_{12} deficient megaloblastic anaemia resulting from gastrectomy [22] and fish tapeworm infestation [15] In these cases, the transketolase activity is not raised [16, 17] as in uncomplicated pernicious anaemia. This probably reflects a subclinical thiamine deficiency which results in a fall from the raised values associated with uncomplicated megaloblastosis, to within the normal range It is suggested a similar phenomenon occurs in nutritional folate deficiency, a suggestion which is supported by the demonstration that the TPPE was increased in 35% of patients studied

Further evidence that this is so is provided by the estimation of plasma thiocyanate levels in pernicious anaemia and folate deficiency The thiocyanate ion is found in the same foodstuffs as folate polyglutamates and is a useful index of nutritional status provided the subjects do not smoke and renal function is normal In pernicious anaemia, the mean level did not differ from age-matched controls while in folate deficiency [25] the mean level was significantly lower than the control values.

Table 1 Fasting blood pyruvate, lactate and oxoglutarate levels in 8 patients with pernicious anaemia and 4 with folate deficiency

Haemoglobin, g/100 ml	RBC/mm³	Transketolase activity, IU/l	Thiamine pyrophosphate effect, %	Serum folate, ng/ml	RBC folate, ng/ml	Fasting blood pyruvate, mg/100 ml	Fasting blood α oxoglutarate, mg/100 ml	Fasting blood lactate, mg/100 ml
				Pernicious anaemia				
11.6	–	77	15	14.3	253	0.86	0.10	–
11.1	2.9	96	8	25	600	1.05	0.09	3.8
7.7	2.1	98	18	12	240	0.57	0.19	3.8
8.3	2.8	129	18	8.2	126	0.805	0.13	–
5.4	1.3	74	36	3.8	40	0.99	0.25	11
7.2	1.9	91	35	9.0	122	0.62	0.12	–
6.3	–	112	20	6.5	500	0.80	0.12	10.5
14.0	4.7	76	19	3.0	100	1.05	0.09	–
				Folate deficiency				
6.2	1.6	57	21	1.8	40	1.37	0.21	7.6
5.5	1.3	31	42	2.8	30	1.84	0.18	8.1
3.4	1.5	69	39	1.2	62	0.81	0.14	5.1
5.4	1.7	24	49	1.2	55	0.96	0.14	9.2

Blood transketolase levels are sometimes low in thyrotoxicosis compared with healthy controls [13, 17], and this probably also reflects an associated vitamin B_1 deficiency This group of patients are also often folate deficient and excrete increased quantities of formiminoglutamic acid [21]

Since the TPPE is only rarely increased in pernicious anaemia whether attended by neuropsychiatric symptoms or not, it is concluded that B_1 deficiency is not a feature of this disease and plays no part in the genesis of the hyperpyruvicacidaemia observed by previous workers [3, 8, 12] Raised fasting pyruvate levels were also noted in anaemia of nutritional folate deficiency *It may well be that raised pyruvate levels in these 2 deficiency states reflect interference* with the conversion of pyruvate to acetate which, in *Clostridium thermoaceticum* at least requires both vitamin B_{12} and folate as essential co-factors [14]

The increased red cell transketolase activity seen in patients with an iron deficiency anaemia responding to treatment, in most patients with congenital haemolytic anaemia and in some with acute leukaemia suggests that transketolase activity is normally greater in young erythrocytes than in older cells This latter point has been confirmed by estimating the transketolase activity in red cell preparations of different ages that had been separated by differential centrifugation.

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Acta Haemat 47 225-232 (1972)

Congenital Methemoglobinemia Due to Hemoglobin M

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Abstract Five cases of congenital cyanosis due to hemoglobin M_{Iwate} in 2 generations of one family are reported Another case of hemoglobin M with cyanosis is mentioned The patients did not have any symptoms related to the cardiac pulmonary or hematologic systems, and had always led active lives Slight but definite cyanosis was most pronounced in their lips and fingertips.

Key Words
Hemoglobin M
Hemoglobinopathies
Hereditary methemoglobinemia

Methemoglobinemia usually occurs as an acquired disorder following the administration of oxidative drugs or compounds [1], but it is clearly established that the non-acquired type of methemoglobinemia is a molecular disease The hereditary types of this disorder may arise either from the diminished reconversion of methemoglobin to hemoglobin, or from an abnormality of the globin section of the hemoglobin molecule which allows the prosthetic groups to exist only in a ferric state

Recently a family, in which 5 members from two generations had congenital methemoglobinemia due to Hemoglobin M_{Iwate} was studied in this department Another adolescent girl with congenital cyanosis due to hemoglobin M was also observed Since hereditary methemoglobinemic cyanosis due to HbM is a fairly rare condition which has never been reported from Turkey, we thought it worthwhile publishing our findings

Case Reports

Family F The propositus was a 21 year-old Turkish male who was referred to the Hematology Clinic from another hospital because of generalized cyanosis since birth. He had no complaints apart from excessive sweating and cyanosis.

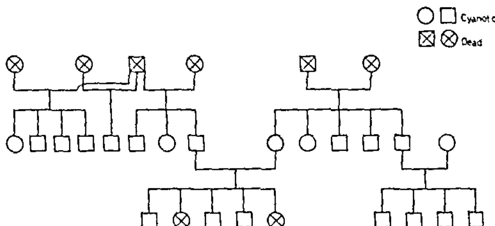


Fig 1 Family tree of the patient with HbM_{Iwate}. The propositus is the one cyanotic square at bottom left

Physical examination revealed a well developed well nourished man with marked cyanosis which was most pronounced in the lips external ears and nail beds The palpebral conjunctivae were of a brown color there was no clubbing of the fingers The lungs were clear to percussion and auscultation and the heart sounds were of good quality with regular rhythm No murmurs were detected and the heart size was within normal limits The remainder of the examination was unremarkable Blood pressure was 120/80 and pulse rate 80/min Hb 18 75 g%. Hematocrit 52% White blood cell count was 7 800 mm³ with a normal differential

As can be seen from the patient s family tree (fig 1) his mother uncle and 2 of the uncle s sons have cyanosis Since the family lives far from Ankara detailed hematologic studies could not be performed and only blood was obtained for hemoglobin analysis However in all cases physical examination was unremarkable

Family A The proposita a 16-year old Turkish girl was seen in the Hematology Clinic because of generalized cyanosis since early infancy She was an underdeveloped undernourished chronically sick looking white female with pronounced cyanosis of the lips and nail beds There was no clubbing of the fingers and the remainder of the physical examination was non contributory Hb 9 50 g%. Hematocrit 30%.

The patient s mother and 2 brothers were not cyanotic but information about her father and other close relatives was limited Consanguinity between the parents was denied

Methods

Methemoglobin and fetal hemoglobin (HbF) concentrations were determined by the methods of EVELYN and MALLOY [2] and SINGER [3] respectively Erythrocyte reduced glutathione (GSH) glutathione instability glucose 6 phosphate dehydro-

genase and NADH-dependent methemoglobin reductase (diaphorase) activity determinations were performed by the methods of BEUTLER [4], GRUNET and PHILLIPS as modified BEUTLER [5], ZINKHAM [6] and SCOTT with the modification of ROSS [7] successively. Starch gel electrophoresis was performed by SMITHIES method [8] and agar gel according to ROBINSON *et al.* [9]. The hemoglobin level was determined as cyanmethemoglobin. For spectroscopic studies, Beckman's DU spectrophotometer was used, and benzoquinone acetic in the urine was tested according to FISHBERG [10].

Results

At 0.4 and 2%, the concentrations of methemoglobin and HbF in the first patient were within the normal limits of our laboratory values. His erythrocyte G-6PD (169 u), GSH (92 mg%), glutathione stability (77.5 mg%) and NADH-dependent diaphorase activity (39 u = $\triangle$MOD$\times 10^4$/min) were also no different from the control values. Hemoglobin electrophoresis on starch gel (at pH 8.6) and agar gel (at pH 6.45) disclosed no abnormality. When the Hb electrophoresis on starch gel at pH 7 was performed, the abnormal Hb component was visualized on a methemoglobin made specimen (fig. 2). An abnormal component was also shown in the hemolyzate at this pH of the patient's mother, maternal uncle and 2 of the uncle's sons. The absorption spectrum of the propositus was not clearly different from normal acid methemoglobin, but 2 small peaks at 495 and 608 μm could just be discerned in his hemolysates. He was given methylene blue (2 mg/kg) and vitamin C (500 mg) intravenously but showed no recovery from cyanosis. Benzoquinone acetic acid could not be found in the urine. The hemolyzates of the patient and some of his relatives with HbM were sent to Dr BRIMHALL and Dr JONES in Oregon, USA, where the presence of HbM was confirmed and the type of this abnormal hemoglobin was identified. Chemical characterization of the hemoglobin was carried out by Dr BRIMHALL and Dr JONES according to the procedures described by JONES *et al.* [11]. The peptide pattern of tryptic peptides from the aminoethylated α-chain exhibited the characteristics of HbM_{Iwate} ($=HbM_{Kankakee}$) and the abnormal component formed approximately 20% of the patient's hemoglobin.

In the second patient, the presence of HbM was only shown by starch gel electrophoresis at pH 7 with the methemoglobin fraction (fig. 3). Neither the starch gel electrophoresis at pH 8.6 nor agar gel at

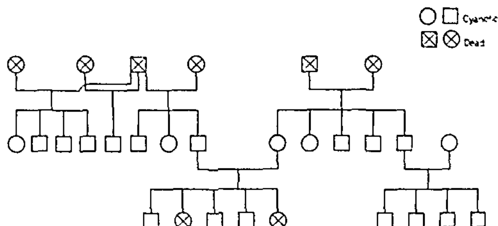


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Fig 3 Hemoglobin electrophoresis of the second patient on starch gel at pH 7 The patient's HbM fraction is well separated (at the bottom) on the methemoglobin

in the literature there are at least 2 cases of HbM where no one else in the patient's family had had cyanosis [13] neither could any history of congenital cyanosis be obtained from among the relatives of our second patient The 2 families studied lived more than 500 miles apart from each other, so any relationship between them was out of question The type of HbM was not identified in the second patient

The hemoglobin variants which together are labeled HbM are a special group of abnormal hemoglobins, which is the only one to show uniquely successful separation in the methemoglobin form from HbA on electrophoresis at pH 7 [14] In these cases HbM diagnosis was first made at this pH on starch gel

So far only the heterozygous state of this abnormal hemoglobins has

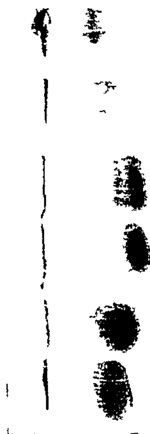


Fig 2 Hemoglobin electrophoresis on starch gel the control s oxyhemoglobin (upper two) methemog methemoglobin (lower two) specimens are shown H patients methemoglobin fraction

pH 6 45 showed any abnormal hemoglobin hemolyzate HbM was found neither in her ers hemolyzates Her methemoglobin (usin and HbF concentrations were 0 1 and 2 1° no further studies could be made on this pat

Comment

Methemoglobinemia due to the pres globinopathies, is inherited as a domin

Acknowledgement I am indebted to Dr BREMHALL and Dr JONES of the University of Oregon Medical School for their kindness in analyzing the HbM of the first family

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Acta Haemat 47 233–237 (1972)

Circulating Plaque-Forming Cells after Transfusion

H.-W. Baenkler

Department of Clinical Immunology, University Hospital
(Director Prof. Dr. F. Scheiffarth), Erlangen

Abstract. Prior to, and 12 to 14 days after, transfusion lymphocytes (L) and white blood cells (WBC) were isolated from venous blood samples. Release of antibodies directed against red blood cells (RBC) of the donor were demonstrated by local hemolysis in gel. Controls were done with RBC of the recipient. The results show a significant increase of antibody releasing cells after transfusion in L. A significant increase among WBC could not be found. Results demonstrate a possible sensitization against transmitted RBC even if serological tests are negative.

Key Words
Blood transfusion
Blood groups
Erythrocyte antigens
Lymphocyte antibodies
Plaque forming cells

The discovery of the blood groups revealed a system of antigenic determinants localized at the surface of the red blood cells (RBC). Besides the first demonstrated ABO system, some other systems, such as Rh, MNSs, etc., have been found. Naturally-existing isoantibodies can be analyzed only in a few systems. By screening tests and cross matching, circulating antibodies can be detected. But even if these tests cannot reveal any risk regarding the transfusion of blood, a sensitization may be possible, since not all know systems are routinely typed. Therefore, antibodies against RBC of the donor occasionally can be found. Thus, it was of interest to look for sensitization against transmitted RBC, using a very sensitive method to detect single antibody-releasing cells when no circulating antibodies could be demonstrated after transfusion. Such cells have been demonstrated by the plaque technique, which shows local hemolysis in gel around the cells.

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Material and Methods

Patients of both sexes between 24 and 69 years of age participated in the study. They were hospitalized because of degenerative or inflammatory diseases. The immune system was not involved. Neither cortisone nor cytostatic or other immunosuppressive drugs had been previously administered. Fresh or stored blood had never been transfused. Clinical treatment was performed without the mentioned drugs. Before transfusion the following examinations were done: typing of donor and patient (ABO, Rh, Kell), search for atypical antibodies and autoantibodies, cross matching.

Collection of plaque forming cells (PFC). Just prior to transfusion of 500 ml blood and 12 to 14 days after transfusion 40 ml venous blood were collected by puncture and heparinized. After gentle mixing the tubes were left 60 min in an upright position. The supernatant rich in white blood cells (WBC) was separated and divided into 2 parts. One part was used for the plaque technique without further manipulations, the other was filtered through a synthetic gaze to gain lymphocytes (L). WBC and L were washed free of serum with buffered Hanks' solution and counted. Viability was tested with trypan blue. This was done at 4 °C.

Detection of PFC. The plaque technique was performed in the usual way [1, 2] with slight modifications. The isolated WBC and L were separately spread after correction according to the viability into gel containing washed RBC originating from the donor of the transmitted blood. After incubation at 37 °C for 60 min complement (guinea pig serum diluted 1:20 with buffered saline) was added. After further incubation under the same conditions direct PFC, releasing 19S immunoglobulin (DPFC), were detected by local hemolysis (plaques). When antihumanglobulin serum (diluted 1:100 with buffered saline) was added as developing serum before complement, indirect PFC (IPFC), releasing 7S immunoglobulin, could be detected by local hemolysis. Analogous experiments served as controls, and RBC of the patient were used. RBC of the donor and the patient were stored under sterile conditions.

At the same time, the serum of the patient was tested for circulating agglutinating or hemolyzing antibodies against transmitted as well as own RBC.

Results (tables I and II)

Antibody releasing L. The viability of L was about 85%. In 10^6 cells before transfusion, there were no or only 1 DPFC, after transfusion the highest value was 3 cells. Controls before and after transfusion showed either no or just 1 DPFC. Using the indirect technique before transfusion, the count was zero or only 1 IPFC, after transfusion, IPFC reached 5 per 10^6 L. The controls in this experiment showed no or just 1 IPFC before and after transfusion. The increase of DPFC and IPFC, using transmitted RBC, is significant (p0.01) according to the Wilcoxon signed rank test

Table I Plaque forming cells per million WBC

Diagnosis	DPFC				IPFC			
	using transfused RBC		controls		using transfused RBC		controls	
	before transfusion	after transfusion	before transfusion	after transfusion	before transfusion	after transfusion	before transfusion	after transfusion
1 Ulcus ventriculi	4	7	3	4	6	8	4	5
2. Phlebitis	6	7	6	6	8	7	7	5
3 Oesophagitis	4	4	4	5	7	9	5	6
4 Ulcus duodeni	5	6	5	5	5	6	5	6
5 Nephropathia	3	4	6	4	6	8	6	7
6 Ulcus ventriculi	3	3	5	6	3	6	7	4
7 Ulcus ventriculi	5	4	5	5	4	4	4	5
8 Hernia inguinalis	6	5	4	4	5	4	4	6
9 Nephropathia	7	8	6	5	6	7	6	6
10 Arthrosis	5	6	4	7	4	5	5	4
11 Ulcus ventriculi	4	5	5	6	3	3	7	5
12. Ulcus duodeni	5	5	5	6	3	2	7	7
p					–		–	

Table II Plaque forming cells per million L

Diagnosis	DPFC				IPFC			
	using transfused RBC		controls		using transfused RBC		controls	
	before transfusion	after transfusion	before transfusion	after transfusion	before transfusion	after transfusion	before transfusion	after transfusion
1 Ulcus ventriculi	0	2	1	1	1	3	1	1
2. Phlebitis	0	2	0	0	0	2	0	0
3 Oesophagitis	0	1	0	0	0	2	1	1
4 Ulcus duodeni	0	1	0	1	1	3	0	1
5 Nephropathia	0	2	1	0	1	3	0	0
6 Ulcus ventriculi	0	0	0	0	0	2	1	0
7 Ulcus ventriculi	1	2	1	0	0	4	1	1
8 Hernia inguinalis	1	2	1	1	1	4	0	1
9 Nephropathia	1	3	0	0	0	5	0	0
10 Arthrosis	0	0	1	0	0	2	1	0
11 Ulcus ventriculi	1	2	1	1	1	3	1	1
12. Ulcus duodeni	0	2	0	0	1	3	1	1
p	<0.01				<0.01		–	

Antibody-releasing WBC The viability was about 80%. In 10^6 cells, there were, before transfusion, 3 to 7 DPFC, after transfusion, 4 to 8 DPFC Controls had between 3 and 6 DPFC before transfusion and between 4 and 7 DPFC after transfusion Using the indirect technique, there were, at first, between 3 and 8 IPFC, after transfusion, between 3 and 9 IPFC Controls initially had between 4 and 7 IPFC, after transfusion, they had the same counts The statistical analysis according to the Wilcoxon signed rank test showed no significance

The tests for circulating antibodies against transmitted or own RBC were negative in all cases

Discussion

The demonstration of PFC using WBC and L of the recipient and RBC of the donor means an immunologic process The results indicate, however, that besides an immune response, there may be unspecific processes that must be taken into account The appearance of hemolytic plaques around single cells demonstrates that these cells release antibodies directed against antigenic determinants localized at the surface of the transmitted RBC The low count of PFC is in agreement with a weak sensitization Controls with autologous RBC showed, in most patients, less PFC. The fact that less PFC were recruited from L than from WBC is due to the heterogenicity of WBC, which contain granulocytes, monocytes etc This will be discussed later

The antibodies causing hemolysis originate from PFC While this statement is quite obvious, it cannot yet be decided if these cells are in fact producing antibodies

There is no doubt that PFC may circulate in the blood [3], but their capacity to produce antibodies has been denied [4] The counts of PFC in L were low The increase of PFC after transfusion with L is significant regarding DPFC and IPFC In contrast, the same experiments with autologous RBC showed no difference before and after transfusion This means a true sensitization Using WBC, there is no significant difference regarding DPFC and IPFC before and after transfusion Considering this fact and the relatively high count of PFC, it is suggested that there is an unspecific process besides immunologic plaque-formation Probably, some granulocytes rich in enzymes desintegrate and destroy RBC around them Therefore, the counts of hemolytic zones in the population of WBC are the sum of an unspecific and a specific process This also causes a high

count of PFC before and after transfusion with RBC of the donor as well as those of the recipient This nonspecific disintegration masks a significant difference

Postulating antibodies against RBC, it is probable that these are directed against determinants of the membrane Sensitization is, after transfusion, also possible against leucocyte groups or serum proteins of the donor But these substances are not found as the surface of RBC, and, therefore, this interpretation is not valid

The demonstration of a 'background is surprising Using WBC, there may be unspecific disintegration, as mentioned before PFC among L must be interpreted as true immune hemolysis These findings resemble results in other species The real cause is not yet clear In the present experiments, the true immune hemolysis may have been caused by sensitization against substances that aggregated to the membrane of RBC, e g, chemical substances or drugs

The lack of circulating antibodies when PFC can be demonstrated is striking It might be that these antibodies are in a concentration too low for demonstration with serological methods, whereas the local concentration around PFC is high enough to cause lysis of RBC.

Acknowledgements I thank Dipl Ing R NEUWIRTH for the statistical analysis and Mrs BARBARA REGLER for her technical assistance

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Acta Haemat 47 238–242 (1972)

Localization of Erythropoietin in the Glomerulus of the Hypoxic Dog Kidney Using a Fluorescent Antibody Technique

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Abstract Intense fluorescent staining of cells in the glomerular tuft has been demonstrated in a hypoxic dog kidney with an antibody to erythropoietin (ESF) using the indirect fluorescent antibody technique The fluorescent staining in the glomeruli was blocked by a highly purified preparation of ESF This fluorescence appears to be localized in the visceral epithelial cells of the glomerulus Kidney sections of a normal dog and a dog injected with hypoxic dog plasma ESF to maintain a prolonged elevation in plasma ESF titers failed to show positive fluorescent staining following treatment with the antibody to ESF The present studies suggest that the epithelial cells of the glomerular tuft are the site of ESF production

Key Words
Erythropoietin production
Fluorescent antibody technique
Kidney and erythropoietin

Several investigators have shown that the kidney is the primary site of erythropoietin (ESF) production [1–3] Fluorescent antibody studies by FISHER *et al* [4] and FRENKEL *et al* [5] have localized ESF in the glomerular tuft of anemic sheep kidney BUSUTTIL *et al* [6] have also reported a cytological localization of ESF in the epithelial cells of the glomerular tuft in an anemic human kidney using the fluorescent antibody technique The cytoplasmic localization in the epithelial cells of the glomerulus was confirmed by destaining the fluorescein labelled tissue section and restaining for morphological study with light microscopy However, the question still arises at to whether this locus of ESF is the site of production storage, or uptake by the epithelial cells in the capil

[1] Supported by USPHS Grant AM 13211 and a Louisiana Heart Summer Research Fellowship

lary network of the glomerular tuft. This paper reports studies which further support our hypothesis that the localization of ESF in the epithelial cells of the glomerulus does not represent a trapping or uptake phenomenon but is more likely a reflection of production of ESF by these cells.

Materials and Methods

Anti-erythropoietin serum (anti-ESF) was prepared by injecting crude human urinary ESF (75 U/mg protein) into an adult male albino rabbit according to a modification of the method of KABAT and MAYER [7]. Anti-ESF activity was determinded *in vivo* on ^{59}Fe incorporation in red cells of ex-hypoxic polycythemic mice [8]. As seen in table I, 0.1 ml of antiserum blocked completely the effects of 0.2 U ESF on ^{59}Fe incorporation in red cells of polycythemic mice. A 15 kg hematologically normal dog was anesthetized with penotobarbital (30 mg/kg i.v.) and 150 ml of hypoxic dog plasma containing 1.2 U ESF/ml (180 U) was injected into the femoral vein at 0, 1, 2 and 4 h (total 720 U). Samples of blood were collected for determination of ESF titers in plasma initially and after 5 h and the ESF titers are shown in table II. Frozen sections of the kidneys were prepared with a cryostat (-20 to -24 °C), air dried and fixed in 95% ethanol. Frozen sections were treated with anti-ESF serum and incubated in a humidifying chamber at 37 °C for 45 min, rinsed with pH 7.2 buffer and stained with Rhodamine counterstain. The sections were then treated with fluorescein isothiocyanate labelled goat anti-rabbit globulin and incubated at 37 °C for 45 min before being washed with buffer and mounted in glycerin. Kidney sections from a hematologically normal and a posthypoxic dog were compared with the sections from the dog infused with erythropoietin, using the above technique, and examined with a fluorescence microscope.

Table I. Inhibitory effect of ESF antiserum on human ESF in polycythemic mice

Treatment	Number of mice	Mean ^{59}Fe incorporation %
Saline	5	0.33 ± 0.079
0.2 U ESF[1]	5	8.11 ± 1.53
0.2 U ESF plus 0.1 ml anti-ESF[2]	5	0.29 ± 0.044

± = Standard error of mean.

[1] Human urinary ESF.

[2] Rabbit antiserum to ESF (human) was absorbed with a 1:4 dilution of normal human serum.

[3] Specific activity estimated to be 2,000 U/mg protein.

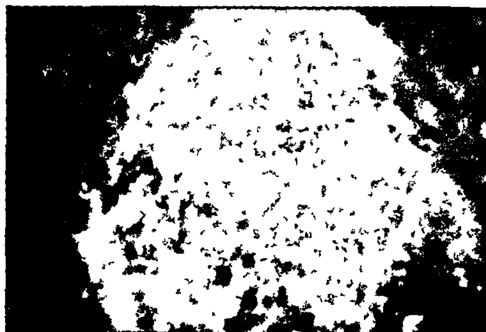


Fig 1 High power fluorescence photomicrograph of a post hypoxic dog kidney section treated with anti ESF serum × 440

Table II ESF activity of dog plasma following injection of ESF[1]

Treatment	Number of mice	ESF U/ml
Saline	5	0
0 1 U ESF	5	0 1
Hypoxic dog plasma	5	1 90
Dog plasma 10 min	5	0 32
Dog plasma 2 h	5	0 26
Dog plasma 4 h	5	0 27
Dog plasma 5 h	5	0 51

[1] 180 U dog plasma ESF injected i v at 0 1 2 and 4 h

Results

Figure 1 shows a high power view of the intense fluorescent staining of a glomerular tuft in the hypoxic dog kidney treated with anti ESF

The specific yellow-green fluorescent staining was only seen in the glomerular tufts and was the most intense in the periphery of the glomerulus. This pattern was quite similar to that described for the anemic human kidney [6]. Hypoxic dog kidney sections treated with either normal rabbit serum or with antiserum which had been previously neutralized with highly purified human urinary ESF did not show any fluorescence. Kidney sections from normal dogs or a normal dog injected with hypoxic dog plasma containing a high titer of ESF were also devoid of fluorescence. The absence of fluorescence in the kidneys of the dog injected with a high dose of ESF suggests that erythropoietin is not taken up by the kidney even when exposed to high titers of the hormone.

Discussion

Our previous report [6] of localization of ESF in the epithelial cell of the glomerular tuft using the fluorescent antibody technique was not completely definitive because it was possible that the epithelial cell could have trapped ESF as blood with high levels of ESF circulated through the kidney. The results of the present study indicate that when plasma levels of ESF are elevated without stimulating kidney production of ESF, the glomerulus does not show positive fluorescence with the use of the fluorescent ESF-antibody. When plasma levels of ESF were increased (1.2 U/ml) in a normal dog for 5 h by intravenous injections of ESF from hypoxic dog plasma, no detectable fluorescence was seen in the glomeruli of the kidney. In contrast, a hypoxic dog kidney revealed intense fluorescence in the epithelial cells of the glomerular tuft when anti-ESF was applied to the kidney using the *fluorescent antibody technique*. These studies suggest that the epithelial cell of the glomerular tuft serves as the site of production of ESF rather than as a site of uptake and storage.

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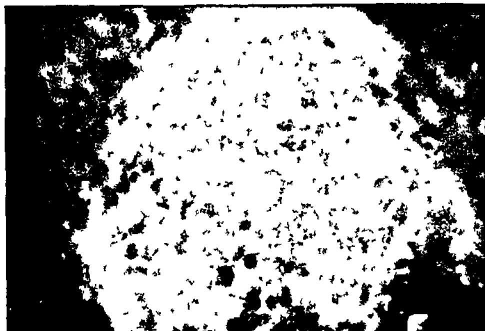


Fig 1 High power fluorescence photomicrograph of a post hypoxic dog kidney section treated with anti ESF serum × 440

Table II ESF activity of dog plasma following injection of ESF[1]

Treatment	Number of mice	ESF U/ml
Saline	5	0
0 1 U ESF	5	0 1
Hypoxic dog plasma	5	1 90
Dog plasma 10 min	5	0 32
Dog plasma 2 h	5	0 26
Dog plasma 4 h	5	0 27
Dog plasma 5 h	5	0 51

[1] 180 U dog plasma ESF injected i v at 0 1 2 and 4 h

Results

Figure 1 shows a high power view of the intense fluorescent staining of a glomerular tuft in the hypoxic dog kidney treated with anti ESF

Acta Haemat 47 243-249 (1972)

Hereditary Hypoprothrombinaemia

True Deficiency of Factor II

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Abstract A 27 year-old man, affected by true deficiency of prothrombin (6%) with a haemophilia like arthropathy is reported The disorder seems to be transmitted as an autosomal recessive The thromboplastin generation test was abnormal in relation to prothrombin deficiency in the substrate plasma A T½ value of 72 h was obtained

Key Words
Arthropathy
Factor II deficiency
Haemarthrosis
Hypoprothrombinaemia

In this paper we report the clinical and laboratory findings of a patient with true deficiency of prothrombin The outstanding clinical feature in this patient as compared to the other reported cases [1–8] is the arthropathy as severe as it is usually observed in haemophilia

Case History

A 27 year-old man was referred to the out patient haematology clinic in April 1969 with the diagnosis of haemophilia His paternal grandfather was a brother of his maternal great grandmother He gave a history of a long series of bleeding episodes since the age of 3 years traumatic bleeding from the inferior lip, repeated spontaneous and traumatic haemarthroses of the left knee and right ankle joints especially in the spring and in the winter, conspicuous and prolonged bleeding after a wound of the left wrist and after dental extractions. At the age of 21 years he had a deep post traumatic haematomata of the right buttock complicated by involvement of the sciatic nerve and paresis of the right inferior limb which regressed only partially The consequent limping probably has contributed to the

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Table I Blood coagulation studies

	Patient	Control
Glass clotting time, min	25	20
Silicon clotting time, min	55	35
Bleeding time (DUKE), min	2	5
Prothrombin time sec	17	12
Partial thromboplastin time, sec	75	50
Stypven time, sec	17	10
Platelet count per μl	190 000	150 000
Prothrombin consumption, %	90	75
Fibrinogen mg%	280	200–400
Factor II 1 stage [10], %	6.5	80–120
2-stage [11], U ml	8	250
staphylocoagulase [12] %	7	100
immunological det [14], % (fig 5)	9	100
Factor V, %	100	
Factor VII %	108	
Factor VIII, %	130	
Factor IX %	92	
Factor X, %	100	
Factor XIII	clot stable at 1 64 pl dilution	clot stable
Fibrinogen disposal products	absent	absent

Table II Thromboplastin generation test (clotting time in seconds)

Al(OH)$_3$ plasma	N	P	P	N	N	N	P	P	N	N
Serum	N	P	N	P	P	N	N	P	N	N
Platelet substitute							–	–	Nplt	Pplt
Substrate plasma	N	P	N	N	P	P	P	N	N	N
	8	17	7	8	15	15	15	8	12	12

N = normal P = patient Plt = platelets

covered throughout the incubation period Bromosulphalein test serum electrophoresis, serum alkaline phosphatase cephalin flocculation, tymol turbidity test cholesterol and bilirubin were normal Vitamin K_1 did not induce any correction

Prothrombin survival time was determined by infusion of 600 ml of fresh normal plasma (10 ml kg body weight) A $T_{1/2}$ of 72 h was obtained Factor II assay in the patient's relatives is reported in table III



Fig 3 Left knee Reduction of the joint space marginal sclerosis secondary alterations particularly evident in the patella



Fig 4 Tibio-tarsic joint Articular surfaces deformed with sclerosis Anterior dislocation of the astragalus and involvement of the astragalus calcaneal joint posteriorly

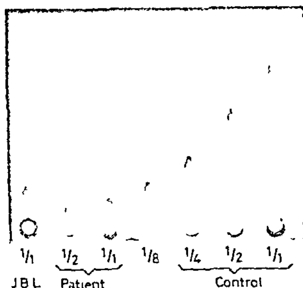


Fig. 5 Immunological determination of factor II. JBL another patient affected by factor II deficiency (12,2%)

Table III Dosage of factor II in the patient's relatives

	1-stage %	Immunological determination %	Staphylocoagulase %
Father	55	57	38
Mother	60	62	46
Sister	49	38	42
Sister	60		-

Comments

It has already been stated that the hereditary form of true deficiency of factor II is transmitted as autosomal recessive [3, 5, 7, 8]. Both parents and sisters of our patient are symptomless, nevertheless they appear

to be heterozygotes with a prothrombin level of approximately 50% of normal Our patient has been haemophilia like syndrome with a factor II concentration of 6% These findings would seem to confirm the transmission as an autosomal recessive character The patient's parents, as in several other cases, are consanguineous [2, 4, 6]

The severity of the arthropathy is another point of interest Haemarthroses may be a feature of congenital hypoprothrombinaemia [1, 3, 5, 8] but permanent joint deformities, as observed by us have not been described Our patient has had normal prothrombin consumption and thromboplastin generation on several occasions The abnormality observed in the TGT seems to be secondary to the prothrombin deficiency in the substrate plasma The patient s plasma and serum behave normally with a normal substrate plasma Others have reported an abnormal TGT [2, 4, 5, 8] The prothrombin T½ is very close to the values available in the literature

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Acta Haemat 47 250–254 (1972)

Leucémie aiguë lymphoblastique chez un sujet atteint d'ataxie-télangiectasie

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Abstract An individual suffering from ataxia telangiectasia (AT) presented an acute lymphoblastic leukaemia He comes from a family in which in addition to the AT which affects half of his siblings, there are a consanguineous marriage and numerous cancers In connection with the present case the authors discuss the main theories as to mechanism of the undoubted facilitating effect of AT on cancer This syndrome is regarded as degenerative malformation of the lymphoid and reticular cells or as an immunological defect

Key Words
Ataxia telangiectasia
Louis Bar syndrome
Lymphoblastic leukaemia

Depuis l'individualisation de l'ataxie-telangiectasie (AT), on a rapporte plusieurs observations de survenue d affection maligne chez des sujets presentant cette affection congenitale Nous versons à ce dossier une nouvelle observation que nous commenterons brievement

Observation

M T (F B 70 907) 23 ans est hospitalisé à la Fondation Bergonié le 19 Mai 1970 alors qu il presente une alteration générale depuis un mois et une épistaxis rebelle au traitement depuis la veille Son état général est mauvais il ne pèse que 32 kg pour 1 60 m Son examen apporte des renseignements de deux ordres

Outre l épistaxis on constate une polyadénopathie superficielle une splenomégalie modéree et une hépatomégalie importante qui évoquent une hémopathie maligne L hémogramme avec 585 000 leucocytes (dont 96% de blastes) par mm³ et le médullogramme permettent de porter le diagnostic de leucémie aiguë lymphoblastique

Par ailleurs existent d une part des télangiectasies oculaires bilatérales caractéristiques et d autre part un syndrome neurologique fait d une débilité psychique et

d'une atteinte cérébelleuse. L'ensemble permet de porter le diagnostic d'AT. Ces troubles sont apparus vers l'âge de 7 ans; ils ont conduit à l'interruption de la scolarité à 10 ans et réduisent ce sujet à un état grabataire depuis une dizaine d'années. Le dosage pondéral des immunoglobulines par la méthode de FAHEY et la recherche des isohémagglutinines ont donné des chiffres normaux. L'étude de l'hypersensibilité retardée n'a pas été faite en raison de la mise en route en urgence du traitement antileucémique.

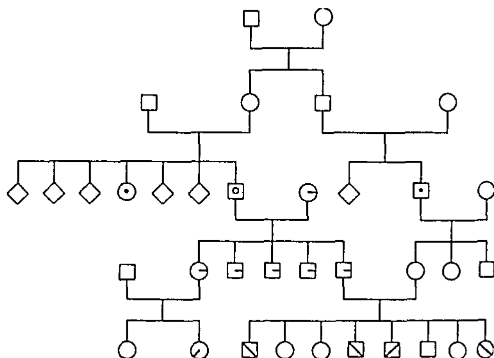
Après une brève période d'amélioration obtenue sous l'influence du traitement associant transfusions et chimiothérapie (prednisone + vincristine + daunorubicine) l'état clinique et hématologique s'aggrave à nouveau et le sujet décède le 16 juin.

La nécropsie a permis de retrouver des infiltrats leucoblastiques intéressant le foie, la rate, l'ensemble des territoires ganglionnaires, l'appendice, la moelle osseuse, les poumons et les surrénales. Il n'a pas été retrouvé de reliquat thymique macroscopique. L'examen neuropathologique, pratiqué après inclusion, a montré d'importantes altérations du cervelet (atrophie corticale de type cérébellifuge) et une atteinte modérée de la moelle (altérations des neurones des cornes antérieurs, pâleur des cordons postérieurs et des racines antérieures et postérieures). Ces altérations du névraxe confirment le diagnostic d'ataxie télangiectasie.

Etude familiale. Nous avons pu reconstituer l'arbre généalogique de ce patient jusqu'à la quatrième génération (fig. 1). Nous relevons en particulier l'existence d'un mariage consanguin favorisant l'expression d'une tare autosomique récessive comme celle dont dépend l'AT. Dans la fratrie de notre malade 4 enfants sur 8 présentent cette tare. Enfin, l'on relève de nombreux cancers, notamment chez les deux grands parents cousins germains et une maladie de Hodgkin chez une cousine au premier degré.

Commentaires

Nous n'insisterons pas sur les caractères de l'AT présentée par notre malade, d'une part parce que près de 200 observations ont été publiées, d'autre part parce que l'exploration, notamment immunologique, de notre cas est restée très rudimentaire en raison de la gravité de la leucémie associée qui a mobilisé toute l'attention. Nous évoquerons seulement les problèmes que posent la survenue d'un cancer sur un terrain présentant à la fois des malformations congénitales et un déficit immunitaire. Cette association est en effet fréquente et l'on peut préciser, après revue de la littérature, qu'environ 15% des sujets atteints d'AT présentent une évolution tumorale intéressant dans presque tous les cas les lignées lymphoïdes ou réticulaires. Il s'agit, le plus souvent, de lymphosarcome ou de réticulosarcome, plus rarement de maladie de Hodgkin ou, comme dans notre cas, de leucémie aiguë lymphoblastique [1, 6, 9, 11]. On retrouve en outre une incidence élevée de cancers dans les familles de ces sujets [4



sexe masculin

sexe féminin

sexe inconnu

cancer gastrique

cancer du foie

hypertension artérielle

maladie de Hodgkin

ataxie-télangiectasie

leucémie aiguë lymphoblastique

9] Ces constatations permettent d'affirmer qu'une telle association n'est pas fortuite et conduisent à proposer des hypothèses explicatives

On peut tout d'abord considérer que l'AT correspond à un terrain génétique particulier, favorisant l'initiation d'une dégénérescence maligne Bien que les malformations nerveuses ne s'accompagnent d'aucune dégénérescence des mêmes tissus, il est tout à fait remarquable que la grande majorité des tumeurs observées concernent les cellules réticulaires et lymphoïdes qui présentent également une malformation dont témoigne l'insuffisance immunitaire Cette observation paraît corres-

pondre à un phénomène plus général. Si le mongolisme, qui est la plus fréquente des malformations diffuses, s'accompagne d'une fréquence accrue de leucémies [12], on observe pour beaucoup d'autres affections congénitales une dégénérescence élective des tissus intéressés par la malformation [10] : tumeurs nerveuses dans la neurofibromatose de Recklinghausen [5], carcinomes cutanés dans le xeroderma pigmentosum [8] ou digestifs dans la polypose rectocolique [2], pour ne citer que quelques exemples parmi les plus évocateurs. Si ces constations sont tout à fait probantes, il ne paraît cependant pas possible de préciser la nature des relations entre la malformation et la dégénérescence : anomalie chromosomique favorisant l'apparition de mutations spontanées, perturbations de la différenciation cellulaire ou fragilité vis à vis de carcinogène exogène?

Par ailleurs, on peut considérer l'AT en tant que syndrome de défaillance immunitaire. Sans vouloir forcément adhérer à la thèse de la «surveillance immunologique» de BURNET [3], on doit reconnaître que de nombreux déficits immunologiques congénitaux ou acquis avérés comme l'agammaglobulinémie de BRUTON et l'AT ou seulement suspectés s'accompagnent de la survenue fréquente de lymphoréticulopathies malignes [7]. L'interprétation de ces faits n'est pas univoque : l'insuffisance immunitaire peut notamment autoriser la pénétration d'un agent oncogène qui aurait été normalement éliminé ou l'émancipation d'un clone cellulaire cancérisé qui se développe malgré une néo-antigénicité probable, à la faveur de la baisse de vigilance des dispositifs cellulaires chargés de faire respecter l'intégrité de l'organisme.

Quel que soit le mécanisme invoqué, son mode d'action dans les phénomènes de la cancérisation est probablement variable tant il est vrai que la dégénérescence maligne dérive plus souvent que d'un événement unique d'une succession d'événements élémentaires qui doivent s'associer dans un ordre déterminé pour que le cancer apparaisse. Quoiqu'il en soit de ces spéculations pathogéniques, il apparaît que l'existence d'une première anomalie en favorise naturellement une ou plusieurs autres. De telles associations ou filiations pathologiques font parfaitement ressortir que la mécanique génétique des mammifères supérieurs présente une fragilité qui est la rançon de son extrême complexité et de son excellente précision en temps normal.

Résumé

Un sujet atteint d'ataxie télangiectasie (AT) a présenté une leucémie aiguë lymphoblastique. Il est issu d'une famille où l'on note, outre l'AT qui touche la moitié

de sa fratrie, un mariage consanguin et de multiples cancers A propos de cette observation, les auteurs rappellent les principales hypothèses permettant d expliquer qu un cancer soit indiscutablement favorisé par l AT, que l on considère ce syndrome comme une malformation des cellules lymphoïdes et réticulaires qui dégénèrent ou comme une insuffisance immunitaire

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VIII World Congress of Anatomic and Clinical Pathology

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Pre-Congress address WAPS VIII World Congress c/o Dr H LOMMEL, Postfach 37, *D-5090 Leverkusen* (Deutschland)

Immunohematology and Hemotherapy

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The programm which will be presented in the auditorium of the University of Texas M D Anderson Hospital and Tumor Institute at Houston, 6723 *Bertner*, Houston, Texas on *September 21 and 22 1972* is divided into 4 parts (1) Basic Immunology (2) Diagnostic Immunohematology, (3) Applied Immunohematology, and (4) Hemotherapy The first day of the program will be completed with a panel discussion of difficult cases in blood banking, and the second day will close with an open forum of questions and answers with the entire *faculty* participating Guest speakers include PETER D ISSITT, LAWRENCE D PETZ, GEORGE GARRATTY plus an outstanding local faculty

For further information write to The Office of the Dean, The University of Texas Graduate School of Biomedical Sciences at Houston, Division of Continuing Education P O Box 20367, *Houston TX 77025* (USA)

Elliptocytosis and Thalassaemia

To the Editor In the paper entitled 'Hereditary elliptocytosis associated with β thalassaemia and a variant of Rh (D) by NAGARATNAM *et al*, which appeared in this journal [*46* 232, 1971] a Sinhalese family with this disorder is reported According to the haematological findings in this family, there was no mutual enhancement of the effects of the particular genes responsible for hereditary elliptocytosis and β thalassaemia In addition to the family reported by PERILLIE and CHERNOFF cited in the above mentioned paper, we also have described a Turkish family with the combination of hereditary elliptocytosis and heterozygous β thalassaemia suggesting a mutual enhancement of the effects of these particular genes [J med Genet *5* 298, 1968] In this paper and also in another [Ann N Y Acad Sci *165* 13 1969] we classified this haemoglobinopathy into 2 types (1) the combination of hereditary elliptocytosis and heterozygous β thalassaemia without enhancement of the effects of the involved genes, and (2) the combination of hereditary elliptocytosis and heterozygous β thalassaemia with either a summation of the clinical effects of the genes or mutual enhancement of any of the involved genes This may be explained by the existence of more than one kind of genes for β thalassaemia and hereditary elliptocytosis

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Environmental Health Aspects of Lead

Symposium Amsterdam, October 2–6, 1972

Topics Uptake and metabolism of environmental lead by man including the use of animal experiments aimed at the establishment of human metabolic models

Subclinical effects of lead Experience gained from lead industrial hygiene and toxicological studies applicable to the better understanding of the biological effects Physiological, morphological and biochemical changes in man in relation with lead uptake Combination effects of lead exposure together with other toxic substances and environmental agents The nature, role and significance of the subclinical effects of lead in establishing lead environmental standards

Epidemiological studies Identification of susceptible human population groups exposed to environmental lead Current epidemiological studies in relation to the health aspects of environmental lead

Secretariat Direction Protection Sanitaire, Commission des Communautés Européennes 29, rue Aldringen *Luxembourg* (Grand Duché)

Acta Haemat. 47: 257-263 (1972)

Effects of Chloramphenicol on Heme Synthesis

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Abstract. The *in vitro* effect of chloramphenicol (CAP) on ferrochelatase (heme synthetase) activity was tested on bone marrow obtained from patients with and without evidence of CAP toxicity. In the control group of 24 randomly selected patients who had not previously received CAP, inhibition of heme synthesis by CAP (30 μg/ml) was found in 2 and stimulation in 4 instances. In the majority, i.e. 18 patients, heme synthesis was not influenced by CAP. Studies on bone marrow obtained from 8 patients who tolerated CAP without adverse effects revealed stimulation of heme synthesis in 2 instances and inhibition in none. Transient inhibition of ferrochelatase was found in only 1 out of 4 patients recovering from aplastic anemia caused by CAP. However, inhibition of ferrochelatase was repeatedly observed in samples obtained from 3 out of 5 patients with reversible, sideroblastic anemia due to CAP. The intensity and incidence of *in vitro* alterations of heme synthesis by CAP increased at higher CAP concentrations (300 μg/ml). We conclude that a direct, dose-dependent interference of CAP with ferrochelatase may inhibit heme synthesis *in vitro* and erythroid iron utilization in susceptible individuals.

Key Words
Aplastic anemia
Chloramphenicol toxicity
Ferrochelatase
Heme synthesis
Sideroblastic anemia

Chloramphenicol (CAP) may cause fatal bone marrow aplasia, but more frequently produces mild and transitory anemia [1]. Conflicting results have been reported concerning the ability of this drug to inhibit cytoplasmic protein synthesis by mammalian cells [2, 3]. However, the specific inhibition of mitochondrial protein synthesis has been well established [4, 5]. Therefore, some of the clinical side effects of CAP, such as reversible bone marrow depression, erythroid iron overload, or aplastic anemia, have been attributed to an interference of the drug with mitochondrial protein synthesis [6, 7], but conclusive evidence is still

Elliptocytosis and Thalassaemia

To the Editor In the paper entitled 'Hereditary elliptocytosis associated with β thalassaemia and a variant of Rh (D) by NAGARATNAM *et al*, which appeared in this journal [*46* 232, 1971] a Sinhalese family with this disorder is reported According to the haematological findings in this family, there was no mutual enhancement of the effects of the particular genes responsible for hereditary elliptocytosis and β thalassaemia. In addition to the family reported by PERILLIE and CHERNOFF cited in the above mentioned paper, we also have described a Turkish family with the combination of hereditary elliptocytosis and heterozygous β thalassaemia suggesting a mutual enhancement of the effects of these particular genes [J med Genet *5* 298, 1968] In this paper and also in another [Ann N Y Acad Sci *165* 13, 1969] we classified this haemoglobinopathy into 2 types (1) the combination of hereditary elliptocytosis and heterozygous β thalassaemia without enhancement of the effects of the involved genes, and (2) the combination of hereditary elliptocytosis and heterozygous β thalassaemia with either a summation of the clinical effects of the genes or mutual enhancement of any of the involved genes This may be explained by the existence of more than one kind of genes for β thalassaemia and hereditary elliptocytosis

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Environmental Health Aspects of Lead

Symposium Amsterdam, October 2–6, 1972

Topics Uptake and metabolism of environmental lead by man including the use of animal experiments aimed at the establishment of human metabolic models

Subclinical effects of lead Experience gained from lead industrial hygiene and toxicological studies applicable to the better understanding of the biological effects Physiological, morphological and biochemical changes in man in relation with lead uptake Combination effects of lead exposure together with other toxic substances and environmental agents The nature, role and significance of the subclinical effects of lead in establishing lead environmental standards

Epidemiological studies Identification of susceptible human population groups exposed to environmental lead Current epidemiological studies in relation to the health aspects of environmental lead

Secretariat Direction Protection Sanitaire Commission des Communautés Européennes 29, rue Aldringen, *Luxembourg* (Grand Duché)

Acta Haemat 47 257-263 (1972)

Effects of Chloramphenicol on Heme Synthesis

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Abstract. The *in vitro* effect of chloramphenicol (CAP) on ferrochelatase (heme synthetase) activity was tested on bone marrow obtained from patients with and without evidence of CAP toxicity. In the control group of 24 randomly selected patients who had not previously received CAP, inhibition of heme synthesis by CAP (30 μg/ml) was found in 2 and stimulation in 4 instances. In the majority, i.e. 18 patients, heme synthesis was not influenced by CAP. Studies on bone marrow obtained from 8 patients who tolerated CAP without adverse effects revealed stimulation of heme synthesis in 2 instances and inhibition in none. Transient inhibition of ferrochelatase was found in only 1 out of 4 patients recovering from aplastic anemia caused by CAP. However, inhibition of ferrochelatase was repeatedly observed in samples obtained from 3 out of 5 patients with reversible sideroblastic anemia due to CAP. The intensity and incidence of *in vitro* alterations of heme synthesis by CAP increased at higher CAP concentrations (300 μg/ml). We conclude that a direct, dose-dependent interference of CAP with ferrochelatase may inhibit heme synthesis *in vitro* and erythroid iron utilization in susceptible individuals.

Key Words
Aplastic anemia
Chloramphenicol toxicity
Ferrochelatase
Heme synthesis
Sideroblastic anemia

Chloramphenicol (CAP) may cause fatal bone marrow aplasia, but more frequently produces mild and transitory anemia [1]. Conflicting results have been reported concerning the ability of this drug to inhibit cytoplasmic protein synthesis by mammalian cells [2, 3]. However, the specific inhibition of mitochondrial protein synthesis has been well established [4, 5]. Therefore, some of the clinical side effects of CAP, such as reversible bone marrow depression, erythroid iron overload, or aplastic anemia, have been attributed to an interference of the drug with mitochondrial protein synthesis [6, 7], but conclusive evidence is still

lacking. RUBIN *et al.* [8] demonstrated that early CAP toxicity can be detected by a delayed incorporation of iron into hemoglobin, even before the appearance of other hematological abnormalities of the peripheral blood.

Previous results from this laboratory [9] revealed that reversible erythroid dysfunction caused by CAP is frequently accompanied by the presence of abnormal sideroblasts in the bone marrow, as well as by an increase in the concentration of erythrocyte protoporphyrin. Reversible sideroblastic anemia associated with CAP administration was also observed by other authors [10, 11], who detected by electron microscopy the presence of *iron deposits within erythroid mitochondria*. The poor utilization of iron and protoporphyrin for hemoglobin synthesis and their subsequent accumulation within red cell precursors suggest that this form of sideroblastic anemia is due to impaired heme synthesis. Such a defect could result either from a reduced synthesis of globin or of ferrochelatase (heme synthetase), or from a direct interference of CAP with iron incorporation into protoporphyrin. We investigated this latter possibility by measuring *in vitro* the ferrochelatase activity of human bone marrow cells in the presence of therapeutic as well as toxic concentrations of CAP (30 and 300 μg/ml, respectively). A preliminary report of these findings has been presented [12].

Methods

Ferrochelatase activity was monitored by measuring the amount of ferrous ^{59}Fe incorporated into protoporphyrin within 30 min by 10^8 nucleated erythroid precursors according to the method of BOTTOMLEY [13]. The difference between iron incorporation into isolated and crystallized heme [14] by cell lysates incubated with or without CAP was compared and analysed for statistical significance by Student's t test. Each enzyme assay was carried out in tri- or quadruplicate.

Results

A first control group consisted of bone marrow samples obtained from 24 patients, hospitalised mostly for elective surgery, who had not previously received CAP. At the concentration of 30 μg/ml, CAP did not affect the rate of heme synthesis in the majority of these cases, including one patient suffering from idiopathic sideroblastic anemia (table I). In 4 instances, enzyme activity was slightly enhanced (16 ± 7% over

Table I Effects of CAP on heme synthesis *in vitro* Patients not treated with CAP

CAP concentration µg ml	Total number of patients	Number of patients showing		
		no effect	stimulation	inhibition
30	24	18	4	2
300	17	8	5	4

Table II Effects of CAP on heme synthesis *in vitro* Patients treated with CAP without adverse effects

CAP concentration µg ml	Total number of patients	Number of patients showing		
		no effect	stimulation	inhibition
30	8	6	2	0
300	8	5	3	0

control values, range 10 to 25% , $2p \leq 0.1$) In 2 cases, CAP inhibited heme sythesis respectively by 19% ($2p < 0.001$) and by 37% ($2p < 0.01$) A tenfold increase in the concentration of CAP resulted in a higher incidence of both inhibition and stimulation and produced greater effects.

The second control group included 8 patients previously treated with CAP without developing signs of bone marrow dysfunction. Marrow samples were obtained from these patients within 4 days to 34 months after the end of CAP therapy (table II) In 2 cases, stimulation of enzyme activity was obtained ($2p < 0.01$), the percentage of enhancement amounted to 12 and 56% with 30 µg ml of CAP and to 17 and 106% with 300 µg ml. In a third case, stimulation occurred only at the higher dose of CAP (19%, $2p = 0.01$) Inhibition of heme synthesis was not observed within this group using either concentration of the drug

We tested also 4 patients who recovered from aplastic anemia due to CAP (table III) In 3 instances, CAP had no effect on the amount of heme synthesized One patient was tested on 2 occasions following complete recovery from severe aplastic anemia. The first investigation revealed a marked inhibition of heme synthesis by CAP However, 8 months later, ferrochelatase activity was no longer depressed by the drug

Table III. Inhibition of heme synthesis *in vitro* by 30 µg/ml CAP. Patients who recovered from bone marrow aplasia caused by CAP

Name	Age	Interval between CAP treatment and test, months	Inhibition
1. J.R.	15 months	4	45% (2 p = 0.001)
	23 months	12	nil
2. W.H.	55 years	19	nil
3. P.G.	35 years	26	nil
4. T.C.	7 years	37	nil

Table IV. Inhibition of heme synthesis *in vitro* by CAP. Patients who developed reversible sideroblastic anemia during CAP therapy

Name	Age	Interval between CAP treatment and test	Extent of inhibition, % of control values			
			30 µg/ml CAP		300 µg/ml CAP	
			%	2 p	%	2 p
1. H.E.	85	during treatment	–	–	87	0.05
		6 days	54	0.07	83	0.001
2. S.F.	60	during treatment	28	0.02	28	0.001
		11 days	5	0.05	20	0.005
3. B.C.	47	during treatment	nil		nil	
		13 days	nil		nil	
4. C.M.	75	21 months	nil		nil	
5. G.S.	33	31 months	37	0.001	–	–

The last group included 5 patients who recovered from sideroblastic anemia caused by CAP (table IV). Three patients were tested twice, both during and after CAP therapy. The results of the tests performed on the first 2 patients showed that CAP, at the 2 concentrations used, significantly inhibited heme synthesis *in vitro*. A third patient (G.S.) was tested almost 3 years after treatment; in spite of this long delay, ferrochelatase activity was still markedly depressed by CAP (30 µg/ml). However, for the 2 remaining patients (B.C. and C.M.) no effect could

Table V. Inhibition of heme synthesis *in vitro* by 30 µg/ml CAP. Summary of incidences

Patients groups	Inhibition	Stimulation
1 Control group	2/24 (8%)	4/24 (17%)
2 Non-toxic group	0/8 (0%)	2/8 (25%)
3 Aplastic anemia	1/4 (25%)	0/4 (0%)
4 Sideroblastic anemia	3/5 (60%)	0/5 (0%)

be detected with either concentration of the drug. The incidence of heme synthesis inhibition by CAP *in vitro* among the 4 groups of patients studied is summarized in table V.

Discussion

There appears to be a significant correlation between the clinical signs of sideroblastic anemia caused by CAP and the frequency of inhibition of ferrochelatase activity by CAP *in vitro*. In the group of patients who tolerated CAP without adverse effects, the incidence of *in vitro* inhibition is equal to zero. In contrast, 3 out of 5 patients with previous sideroblastic anemia caused by CAP displayed *in vitro* sensitivity to the drug. Consistent inhibition of ferrochelatase by CAP was not observed in the group of patients with previous aplastic anemia. These findings therefore suggest that the 2 patterns of CAP toxicity, i.e. early sideroblastic anemia and the more dangerous aplastic anemia may be caused by different biochemical mechanisms. The incidence of ferrochelatase inhibition in the first control group (8%) correlates well with the frequency of sideroblastic anemia associated with CAP therapy which, according to a prospective clinical study, amounts approximately to 10% (unpublished results). Enhancement of ferrochelatase activity by CAP was seen in a significant number of individuals only among the two control groups. We have at present no explanation for this effect.

One could tentatively explain these results by a structural abnormality of ferrochelatase or of one of its cofactors revealed only in the presence of CAP. While such a mechanism might explain the causal relationship between CAP therapy and early, reversible sideroblastic anemia, it fails to explain other types of CAP toxicity which appear late, such as aplastic anemia. Therefore, this mechanism does not rule out

previous data suggesting a correlation between CAP toxicity and the inhibition of cytoplasmic [2] or mitochondrial [7] protein synthesis by CAP However, our experimental conditions [13] are not suitable for protein synthesis, and ferrochelatase inhibition by CAP cannot be explained by a depression of ferrochelatase synthesis We conclude that in certain individuals who are sensitive to CAP, essentially patients with reversible sideroblastic anemia, one of the pharmacological actions of this drug consists of direct interference with ferrochelatase activity

Acknowledgements This study was supported by the Rudolf R Nissen Foundation and the grant No 3 123 69 of the Swiss National Foundation The skillful technical assistance of Miss SUSAN IRVINE is gratefully acknowledged Dr J SARTORIUS Basle and Dr B KUBANEK, Ulm Germany kindly provided bone marrow samples from their patients with previous aplastic anemia

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Acta Haemat. 47: 264–268 (1972)

Effect of Serum Vitamin B_{12} Binding on Intrinsic Factor Antibody Detection in Pernicious Anaemia

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Abstract. In 42 normal and 65 pernicious anaemia (PA) sera, no significant difference has emerged between the B_{12} binding capacity. However, the range of B_{12} binding in PA sera has been found to be much wider, and it is suggested that this may be a source of error in detecting intrinsic factor (IF) antibodies. To eliminate this error, a new modification of the charcoal adsorption method of IF antibody detection is described.

Key Words
Intrinsic factor antibody
Pernicious anaemia
Serum B_{12} binding
Vitamin B_{12}

We became interested in the subject of serum unsaturated vitamin B_{12} binding capacity in patients with pernicious anaemia (PA) while examining current methods of detecting circulating antibody to human gastric intrinsic factor (IF). The detection of the common, or type I, antibody is based on the finding that this antibody prevents the binding of vitamin B_{12} to the IF component of human gastric juice, but not to the non-IF binders in the juice. If the B_{12} binding capacity of gastric juice is appreciably reduced after addition of a test serum, the latter is presumed to contain IF antibody. However, the test serum itself binds a certain amount of vitamin B_{12}. Therefore, some workers, e.g., IRVINE [4], attempt a correction by comparing the B_{12} binding capacity of gastric juice + test serum with that of gastric juice + normal serum. In order to decide whether this correction is necessary, and if so, whether it is adequate, we estimated unsaturated B_{12} binding capacity of a large number of sera from patients with PA and from healthy volunteers.

Methods

Blood was collected from 42 healthy normal adults and 65 patients with PA. The diagnosis in each case of PA had been established on a history of megaloblastic anaemia responding to vitamin B_{12} therapy and without previous gastric surgery; absolute achlorhydria and/or absent, or virtually absent, IF secretion in the gastric juice after maximal stimulation with histamine or pentagastrin and abnormal Schilling test improving significantly with a suitable dose of gastric IF. All patients with PA were receiving regular treatment with intramuscular vitamin B_{12} and were in complete remission. Blood was taken 5 or more days after the last injection of vitamin B_{12}.

All sera were separated within 24 h of collection of blood and stored at −20 °C.

Serum unsaturated B_{12} binding capacity was determined by a slight modification of the method of GOTTLIEB *et al.* [2]. Sequentially to a test tube containing 2.0 ml of physiological saline was added 0.5 ml of test serum and 3.0 ng of ^{57}Co-B_{12} in aqueous solution. After mixing well on a whirlimixer and by repeated inversions, 1 ml of a suspension of 5.25% charcoal in 1% bovine albumin was added and thoroughly mixed. The mixture was centrifuged at 3,500 rpm for 20 min and the supernatant decanted. The radioactivity of the supernatant was counted (in a well type scintillation counter) and compared with that of an isotope standard containing 3 ng of ^{57}Co-B_{12}, made up with physiological saline approximately to the volume of the supernatant. All estimations were carried out in duplicate and the results expressed in ng/ml.

IF antibody was detected by the charcoal adsorption method of GOTTLIEB *et al.* [2] (with minor changes) in 2 parts. In part one 0.1 ml of normal human gastric juice was pipetted into a test tube containing 0.7 ml of physiological saline. 15 ng of ^{57}Co-B_{12} in aqueous solution was added and well mixed on a whirlimixer and by repeated inversions. Free vitamin B_{12} was absorbed out by addition of 2 ml of a 5.25-percent suspension of charcoal in 1% bovine albumin and by centrifugation at 3,500 rpm for 20 min. The supernatant was removed and counted. Part two of the procedure was carried out as part one, except that 0.2 ml of test serum was added to gastric juice before the ^{57}Co-B_{12} solution. The antibody activity of the serum was calculated by comparing the difference in the radioactivity counts of the 2 supernatants with that of an isotope standard containing 15 ng of ^{57}Co-B_{12}, made up with physiological saline to the volume of the supernatants. All estimations were carried out in duplicate and suitable controls, including a known antibody positive serum, a normal serum and charcoal suspension + ^{57}Co-B_{12} solution only were included in each batch of tests. Test sera reducing vitamin B_{12} binding capacity of gastric juice by more than 5 ng/ml of serum were regarded as positive for IF antibody.

Results

IF antibody was not detected in any of the normal sera. 33 of the 65 PA sera were antibody positive. Of the positive sera, 4 had weak antibody activity, arbitrarily defined as less than 20 ng/ml

Table I Serum unsaturated vitamin B_{12} binding capacity (ng/ml)

	Number	Range	Mean ± standard deviation	Statistics
Healthy controls	42	0 77–2 06	1 249 ± 0 278	t 0.2091
PA, total	65	0 03–5 43	1 276 ± 0 83	p<0.90 >0 80
PA with antibody	33	0 03–3 17	1 31 ± 0 79	t 0 3271
PA without antibody	32	0 12–5 43	1 242 ± 0 877	p<0 80 >0 70

The results of serum unsaturated B_{12} binding capacity estimations are recorded in table I

Discussion

LAWRENCE [5], using a ratio of 2 667 ng of vitamin B_{12}/ml of serum, found the serum unsaturated B_{12} binding capacity to range from 0 856 to 1 880 ng/ml (mean, 1 17, SD, 0 308) in 10 PA patients after 4 months of treatment, compared with 1 041 ± 0 317 ng/ml in 50 controls HELLER *et al* [3] reported serum unsaturated B_{12} binding capacity of 1 984 ± 0 321 ng/ml in 12 patients with vitamin B_{12} deficiency (nearly all PA) compared with 1 595 ± 0 335 ng/ml for 16 controls No attempt was made, in the above or in any other reports to our knowledge, to differentiate between the PA sera with and without IF antibody

In our series, a group comparison of unsaturated B_{12} binding capacity showed no significant difference between the control and PA sera, and between the 2 subgroups of PA sera with and without IF antibody

In the context of an IF antibody test, the mean unsaturated B_{12} binding capacity of PA serum (1 276 ng/ml) appears to be small, compared with the vitamin B_{12} binding capacity of normal gastric juice which, in a group of 4 tested by GOTTLIEB *et al* [2] varied from 33 18 to 73 7 ng ml However, there is a wide range of values of unsaturated B_{12} binding capacity in PA sera in our study, and in individual cases, the contribution by PA serum to the total B_{12} binding could be an ap-

preciable source of error. A 'correction', e.g., as used by IRVINE *et al.* [4], appears desirable. But as table I shows, although the means are comparable to the range of unsaturated B_{12} binding capacity, the range of PA sera is much wider than that of control sera, it is unlikely that in a random pairing the B_{12} binding of a normal serum would balance that of the test PA serum. In the IF antibody detection methods of ARDEMAN and CHANARIN [1] and WANGEL and SCHILLER [6], additional determination of B_{12} binding capacity of the test PA serum and normal serum are carried out. These are subtracted from the B_{12} binding capacity of the gastric juice + test PA serum and gastric juice + normal serum, respectively. The drawback of this 'correction' is that it requires 2 additional steps involving radioactivity measurements, with the inevitable counting errors.

We have, therefore, developed a modified system of IF antibody detection in which the PA test serum is used to provide its own B_{12} binding correction without recourse to separate determinations of serum B_{12} binding. The details of the technique are essentially as described above in the methods section with the exception that in part one of the procedure, 0.2 ml of test PA serum is added *after* ^{57}Co-B_{12} has been well mixed with the gastric juice. In this sequence, the test PA serum cannot affect the binding of vitamin B_{12} to IF, as this has already taken place and serves merely as a balance to B_{12} bound to the test serum in part two of the procedure. The difference in the radioactivity counts of the 2 supernatants, i.e., gastric juice + B_{12} + test serum and gastric juice + test serum + B_{12}, provides a measure of IF antibody activity of the test serum. This method is simple, requires a minimal number of radioactive counts and has proved of particular value in detection of weak IF antibodies. On retesting the 65 PA sera in our series with the modified method another 2 sera with weak IF antibody have been found.

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Acta Haemat. 47: 269–276 (1972)

Urinary Iron Excretion in Nephrotic Syndrome

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Abstract. Quantitative studies revealed losses of appreciable amounts of iron in 2 patients with the nephrotic syndrome. Increased urinary iron losses amounting to as much as 20 to 100 times the normal values were observed, but the amount of iron excretion did not exceed 0.5 mg/day. Therefore an anaemia cannot be explained by this iron loss. Furthermore, it is postulated that the ratio iron : transferrin in the serum influences the urinary iron loss in these patients.

Key Words
Iron loss in urine
Nephrotic syndrome
Renal anaemia
Transferrin
Urinary iron excretion

A nephrotic syndrome is characterized by an increase of the daily amount of protein excreted in the urine. An urinary protein excretion, exceeding 100 mg/day, is considered to be pathological. Proteinurias, which all display some degree of primary attack upon the glomerular capillary membranes, occur in the so-called nephrotic syndrome. On examination of patients, having a proteinuria and at the same time an anaemia, the question was raised, if it was possible to explain an anaemia in the nephrotic syndrome by the urinary loss of transferrin and iron. In connection with this proteinuria, we were interested in the relationship between the loss of different proteins, the level of these proteins in the serum, the iron saturation of the serum transferrin and the loss of the total amount of iron [1, 2]. Therefore we decided to observe clinically and chemically 2 patients with nephrotic syndrome for about 1 year in order to obtain data about the relationship mentioned above.

Materials and Methods

All chemicals used were of analytical grade. Polystyrene tubes and bottles were carefully rinsed with bichromate and aqua bidest. The total protein content has

been determined by the biuret method with pure albumin as a standard [3] The different proteins were determined by the radial immunodiffusion technique of MANCINI *et al* [4] Immunodiffusion plates (Partigen) and a standard of stabilized plasma proteins were obtained from Behringwerke AG (Marburg/Lahn) Serum iron was determined with the bathophenantroline method as described [5, 6] The total iron binding capacity of serum (TIBC) was determined by the method of RAMSAY [7] The determination of iron in urine has been described in detail [8] The results of all these determinations were grafically plotted and the correlation coefficient calculated Hemoglobin has been determined by the method of HAINLINE [9] 24 h urines were collected and immediately examined after collection

Case Reports

Patient S Female of 19 years old with an average protein loss of 5 g/day, the Hb was 5–5½ mmol/l A biopsy of the kidney showed a subacute proliferative glomerulonephritis which was still active She was treated with Prednison R (40–50 mg/day) and Imuran (125 mg/day) The daily protein excretion decreased during this treatment from about 6 to 2.5 g/day

Patient N Male of 22 years old the daily loss of total protein content was 13 g, the Hb was 7 mmol/l A biopsy of the kidney showed a chronic glomerulonephritis (rapidly progressive form) and a serious nephrotic syndrome The protein excretion did not change during the treatment with Prednison and Imuran This treatment had to be stopped because of side effects

Results

The mean urinary iron loss of normals and of the 2 patients is shown in figure 1 A great number of observations in urine and in serum, with 2 weeks intervals, were performed The results are shown in the figures 2–5 If the daily urinary iron loss is plotted against the plasma iron concentration (fig 2) we can see that there is a significant positive correlation, in patient N, but this correlation cannot be shown in patient S If the urinary transferrin content is plotted against the daily urinary iron content, we observe a significant positive correlation in patient S, but this correlation is absent in patient N (fig 3) Figure 4 indicates the significant positive correlation, in both patients, between the daily urinary loss and the saturation in serum calculated as iron/to transferrin concentration If the serum saturation percentage iron transferrin is plotted against the days of observation we may see that this ratio in urine and in serum in the course of the time is about similar (fig 5)

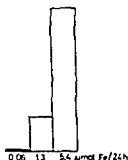


Fig. 1. The amount of iron lost by normals and by the 2 patients. The urinary iron excretion of patient S is about 20 times that of normals and the excretion of patient N is about 100 times that of normal people. The urinary excretion is expressed as µmol Fe/24 h, n means the number of observations made on these subjects.

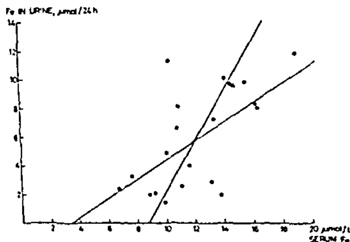


Fig. 2a

All the significant correlations are given together in table I.

In patient S. there is a positive correlation between the urinary iron loss and the serum transferrin concentration and a negative correlation

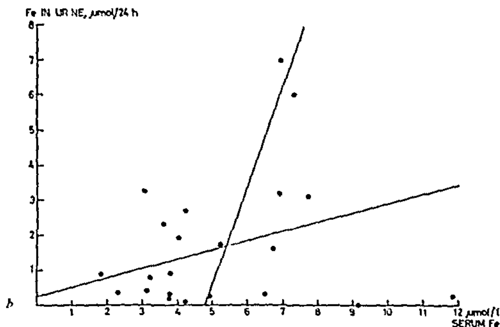


Fig 2 Correlation between the urinary iron excretion, expressed as μmol iron/24 h, and the serum iron concentration (μmol iron/l) *a* Significant positive correlation in patient N ($p<^1/_2$), *b* in patient S there is no correlation as in *a* ($p>5$)

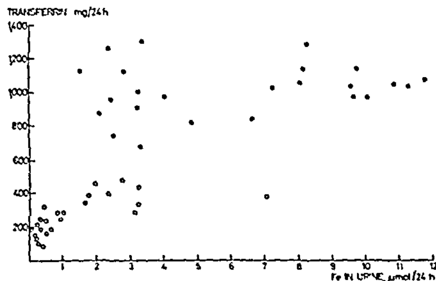
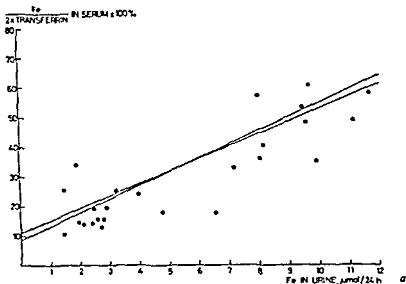


Fig 3 Relation between the urinary transferrin in mg/24 h and the urinary iron loss in μmol/24 h In patient S (o) there is a positive correlation ($1<p<2^1/_2$) This correlation is absent in patient N (•) ($p>5$)



Fe / 2×TRANSFERRIN IN SERUM ×100%

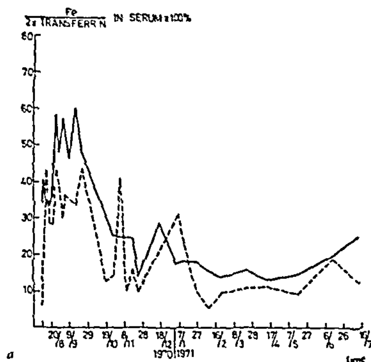
18 16 14 12 10 8 6 4 2

1 2 3 4 5 6 7 8 9 10 11

Fe IN URINE, μmol/24 h

b

Fig. 4. Daily urinary iron loss (μmol/24 h) in relation to the saturation percentage in serum. Fe/2×transferrin means Fe/total iron bound to transferrin calculated as 2× transferrin content both in molar units. In both cases there is a significant positive correlation. *a* Patient N. (p < [illegible]) *b* Patient S. (p = [illegible]).



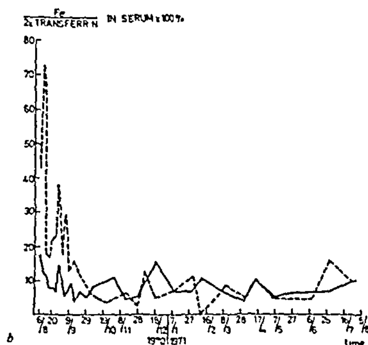


Fig 5 Saturation percentage in urine and serum in relation to time. In both cases, the ratio in urine and in serum is about equal *a* Patient N , *b* patient S Serum (—), urine (---)

Table I

Patient N		Patient S	
Urinary iron —	serum iron; total urinary protein content; TIBC; serum iron transferrin ratio	*Urinary iron* —	urinary transferrin; total urinary protein content; urinary IgG; serum iron transferrin ratio
Urinary iron - - -	total serum protein content; serum albumin; serum transferrin; serum IgG; urinary IgG	Urinary iron - - -	total serum protein content; serum albumin; serum transferrin
		Urinary transferrin - - -	iron TIBC; serum transferrin
		Urinary transferrin	TIBC
Urinary iron transferrin ratio — serum iron transferrin ratio		Urinary iron transferrin ratio — serum iron transferrin ratio	

Full lines mean that a significant positive correlation exists, while dotted lines report a significant negative correlation

between urinary transferrin and serum transferrin In both cases, there is a positive correlation between the ratio iron transferrin in urine and in serum More correlations were investigated but negative results are omitted

Discussion

The purpose of this investigation was to follow the iron excretion of patients with a severe proteinuria. Knowledge about the amount of urinary loss in proteinuria and the amount of serum proteins lost in the urine could possibly give an idea about the cause of the anaemia and the regulation of the iron excretion of these patients. Concerning the amount of iron lost by these patients, we may observe *in figure 1* that the average excretion of iron is 20–100 times higher than in normals. However, an anaemia by an urinary iron loss of not more than 0.5 mg day is diffi-

cult to explain. But in cases of reduced iron stores, a certain degree of an anaemia could be caused by this loss. We expected a significant correlation between the amount of the urinary iron and urinary transferrin and found that in patient S. ($1<p<2^1/_2$). In patient N. a significant correlation did not exist ($p>5$), but in this patient there was a relation between the urinary iron and the serum iron ($p<^1/_2$). The finding of a significant positive correlation between urinary iron and the serum iron : transferrin ratio suggests that this ratio has a regulating effect in the urinary iron excretion in these patients.

The negative correlations between urinary iron and serum proteins levels is explained by the activity of the disease. During an exacerbation of the disease the excretion of protein and, therefore, of iron is increased and the plasma protein content decreased. In case of clinical remissions the reverse may be expected. Since the ratio *iron : transferrin* in urine and serum are about equal, we suggest that the iron is excreted, meanly bound to transferrin.

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Acta Haemat 47 277-282 (1972)

Bone Marrow Return and Division of Circulating Acute Lymphoblastic Leukaemia Cells[1]

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Abstract *In vitro* labelling of circulating blasts with ^{3}H thymidine was followed by their autotransfusion in a case of steady state acute lymphoblastic leukaemia Labelling index and mean grain values were then determined autoradiographically at various intervals on both peripheral and marrow blood The results showed that circulating blasts in S-phase returned to the marrow and then passed back to the circulating compartment after division Their half live values was 22.1 h.

Key Words
Autoradiography
Bone marrow
Leukaemic cells
Lymphoblastic leukaemia

Differences in the kinetic behaviour of acute lymphoblastic leukaemia (ALL) as opposed to acute myeloblastic leukaemia (AML) cells have been reported in recent years [2, 3, 6] The fact that lower marrow than peripheral blast ^{3}H thymidine labelling index (LI) values are occasionally observed in ALL has suggested that leukaemic cells may originate primarily in extramedullary sites in some cases [2] A further deduction from these kinetic differences is that ALL and AML may not share the same stem cell origin [3]

The question of the possible recirculation, survival and colonisation of peripheral blast cells after their release from their genesis sites has not yet been settled Recent studies of autotransfused *in vitro* ^{3}H-cytidine-labelled circulating cells in untreated cases of AML have shown that they are incapable of marrow return [4]

The present paper reports the results of a further experiment designed to study the kinetic behaviour after autotransfusion of *in vitro* ^{3}H thymidine labelled blood in a subject with ALL.

[1] This work was supported by CNR, Rome.

Materials and Methods

Exsanguinotransfusion was employed to withdraw 240 ml of venous blood and inject 100 ml of normal blood over 25 min in an untreated case of clinical and haematological steady state ALL in a 6-month-old girl The withdrawn blood was placed in a Fenwal bag containing ACD solution and incubated at 37 °C with 1 μc/ml ³H thymidine (17 7 c/mM) for 30 min A 50-fold greater dose of cold thymidine was then added and the blood retransfused to the donor over a period of 25 min

White cell counts and formulae were done before withdrawal on the bag blood and on the peripheral and/or marrow blood during and after the reinfusion Peripheral and/or marrow blood smears were prepared on each occasion for the determination of leukaemic blast LI values The smears were fixed in methylic Carnoy and covered with Ilford K2 emulsion After 18 days exposure the preparations were developed and stained with May Grünwald Giemsa buffered at pH 7 4 At different times after exsanguinotransfusion when the percent of labelled cells was low, a maximum of 60 000 cells was examined to determine LI and mean grain count (MGC) Blast survival in the peripheral blood was determined from the halving time ($T^1/_2$) of the number of labelled blasts (N/1 000) with a grain count higher than the mean value after the first halving

$$[\text{survival} = \frac{T}{2}\left(\frac{N}{1\,000}\ \frac{1}{0\,693}\right)]$$

Results

Table I sets out the number of white cells observed in the peripheral blood and in the bone marrow, the blast percentages and their LI and MGC values at the various experimental times An increase in peripheral white cell levels was observed after the exsanguinotransfusion, while a gradual fall to lower than start values was noted during the reinfusion These changes could be due to an initial migration of cells from extravascular districts. It should be noted however, that outset white cell count values were virtually re-established 10 min after the commencement of reinfusion Both peripheral and marrow blast percentages remained constant throughout the experiment, together with bone marrow white cell count The table also illustrates the gradual increase in peripheral LI values during the reinfusion to a final maximum of 0 29% when remixing of the autotransfused blood was complete The MGC at this stage was fully comparable with that observed in the freshly withdrawn blood The later picture included an initial rapid fall in LI values within 5 h accompanied by a strong decrease of the MGC, followed by an increase in the LI to a maximum of 0 41 at the 16th hour and a fur-

le 1 Haematological and kinetic data in a case of ALL before during and after autotransf… peripheral blood incubated *in vitro* with ^{3}H-thymidine

		Peripheral blood				Bone marrow		
		white cell	blasts			white cell	blasts	
		count/ mm^3	%	LI	MGC	count/ mm^3	%	LI
…fore …sanguinotransfusion		42,000	92	3.58		331 000	93	17 52
…g blood		34 700	90	3 65	45 20			
…fter …sanguinotransfusion		72,000	93					
…uring …infusion	5 min	60 000	89	0 051	32.40			
	10 min	43,200	87	0.225	38 85			
	20 min	33,300	91	0.210	47 18			
	25 min	33 000	90					
…fter …infusion	0 h	31 700	89	0.290	45 34	315,000	99	0 0379
	5 h			0 083	30 52			
	12 h			0 211	26 66			
	16 h	25 000	90	0 410	25.30		97	0 100
	22 h			0.210	30 80			
	28 h			0 179	29 90			
	37 h	25 000	92	0.231	21 10			
	46 h			0 109	24.20	342,000	99	0 039
	65 h			0 102	19.30			
	4 days			0 031	26 40			
	5 days	26,000	91	0 010	12.00		99	0 000

ther fall to nil by the 5th day, the MGC remained virtually steady after the initial rapid fall

Labelled blasts were already present in the bone marrow by the end of the reinfusion LI values increased until the 16th hour, and then fell to start levels by the 46th hour and to nil by the 5th day The MGC was initially equal to that observed in the peripheral blood, but later fell much more slowly, without achieving halving by the 46th hour

Figure 1 compares peripheral and marrow LI and MGC. The LI ratio serves to indicate the migration of labelled blasts from one compartment to the other It will be seen that there is a gradual fall in the peripheral marrow LI ratio, whereas the respective MGC ratio remains steady after the slight fall observed at the 16th hour

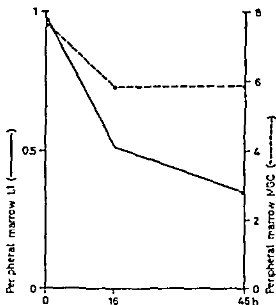


Fig 1 Peripheral marrow LI (——) and MGC (---) ratios at various post reinfusion times

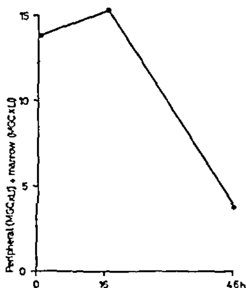


Fig 2 Total blast radioactivity at various post reinfusion times

Specific radioactivity is conventionally expressed in the form, LI×MGC. Since there was no significant change in white cell count and marrow and peripheral blast percent throughout the experiment, the sum

of the 2 radioactivity values expresses the total radioactivity for the two compartments Figure 2 shows that this remained virtually steady until the 16th hour and then fell sharply until the 46th hour

The mean survival of the peripheral blasts was 31 8 h (half-life, 22.1 h)

Discussion

A fall in both LI and MGC values was observed within only 5 h after the reinfusion of labelled peripheral blasts The sharp LI fall can only be attributed to the exodus of these cells from the circulating compartment, whereas the strong decrease of the MGC shows that a certain number of labelled blasts returned to the circulating compartment after a presumably extravasal division (virtual absence of mitotic figures in the peripheral blood) This kinetic pattern was also apparent in the later results for the peripheral blood increase in LI to a value 70% higher than the reinfusion start value by the 16th hour, accompanied by constant MGC from the 16th to the 46th hour

The peripheral picture was thus one of an exit and subsequent re-entry of labelled blasts In the marrow blood, on the other hand, the initially very low LI had increased nearly 3 fold by the 16th hour after the reinfusion Comparison between this early and striking increase and the contemporary fall in peripheral LI values points to an exit of circulating blasts in S-phase followed by their uptake and division in the marrow and their rapid post mitotic return to the circulating district. This interpretation is also supported by observation of significantly higher marrow over peripheral MGC values at the 16th and 46th hour, suggesting that the marrow uptake of circulating blasts was confined to the time required for their mitosis. An inter-district exchange of blasts is also indicated by the virtual steadiness of the total (i e , marrow + peripheral) radioactivity during the first 16 hours. The subsequent fall in total activity is mainly attributable to a fall in compartment LI values, due, presumably, to cell death and to a lesser degree, to a fall in MGC values resulting from occasional further cell divisions. The mean half life of the circulating blasts was about 22 h.

The marrow ward migration and post mitotic release of ALL peripheral blasts is in contrast with the absence of marrow uptake observed following the autotransfusion of ^{3}H-cytidine or ^{3}H uridine labelled peripheral blasts by Hoelzer *et al* [4] and by Clarkson *et al* [1] in AML

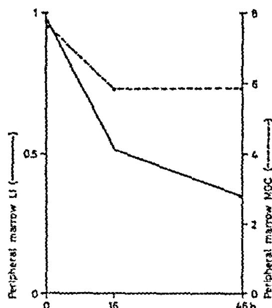


Fig. 1. Peripheral: marrow LI (——) and MGC (----) ratios at various post-reinfusion times

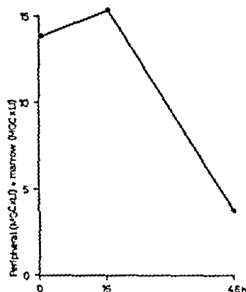


Fig. 2. Total blast radioactivity at various post-reinfusion times

Specific radioactivity is conventionally expressed in the form, LI×MGC. Since there was no significant change in white cell count and marrow and peripheral blast percent throughout the experiment, the sum

Acta Haemat. 47: 283-291 (1972)

Metabolic Activity of Polymorphonuclear Leukocytes in Sickle Cell Anemia[1]

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Abstract. Polymorphonuclear (PMN) leukocytes from patients with sickle cell anemia and a history of repeated infection failed to show a stimulation of respiratory CO_2, O_2 consumption, oxidation of formate and reduction of nitroblue tetrazolium during phagocytosis. The above mentioned abnormalities were not found in PMN leukocytes from patients with sickle cell trait, splenectomized patients and normal individuals. The results of this investigation indicate the presence of an additional predisposing factor to infection in some patients with sickle cell anemia.

Key Words
Infection in sickle cell disease
Leukocyte metabolism
Neutrophil dysfunction
Phagocytosis by leukocytes
Sickle cell anemia
Splenectomy and infection

The infections which are frequently associated with sickle cell anemia may be considered a major complication of this disorder. Most of these patients appear to have experienced some form of infection during the first years of life [8, 13, 19]. Some patients suffer repeated infections during their lifetime [13], while others have no history of infection. Preferential types of bacteria such as salmonella and pneumococcus [12, 13, 19, 22] have been reported as being frequently observed in patients with sickle cell anemia but other microorganisms have also been reported [17, 20]. Predisposing factors such as thrombosis, infarction and necrosis have been considered as being responsible for the increased susceptibility to infection [22]. KAYE and HOOK [16] have suggested that a defect of the

[1] This research was supported by United States Public Health Service Grant CA 11060 from the National Institutes of Health and by General Research Support 7008 from the Philadelphia General Hospital.

reticuloendothelial system inhibits the destruction of phagocytized microorganisms. WINKELSTEIN and DRACHMAN [24] have found marked deficiencies of pneumococcal serum opsonizing activity in patients with sickle cell anemia which prevents adequate phagocytosis and killing of the pneumococcus, thus facilitating overwhelming infection. These authors also consider the nonfunctioning spleen an additional factor contributing to the defective clearance of pneumococci. PEARSON *et al.* [18] demonstrated functional asplenia in children with radioactive technetium. The importance of the spleen for the removal of bacteria from the blood and the increased susceptibility of asplenic patients to infection was reconsidered by these authors. The above mentioned research has provided a more complete understanding of the predisposing factors to infection in patients with sickle cell anemia, but many unanswered questions remain.

The polymorphonuclear (PMN) leukocyte is a major factor in the host defense mechanism against bacterial invasion. Information concerning the phagocytic function of PMN leukocytes in sickle cell disease has not been reported as yet. In this study the metabolic activity of PMN leukocytes from patients with sickle cell anemia was studied during phagocytosis. *There is evidence that PMN leukocytes from patients with sickle cell anemia and a history of infection (HI) possess a metabolic defect which probably interferes with the destruction of material ingested during phagocytosis.* This defect was not evident in PMN leukocytes from patients with sickle cell anemia and no history of infection, patients with sickle cell trait, splenectomized patients and normal individuals.

Methods

Blo[illegible] obtained from pat[illegible] sickle cell anemia, sickle cell trait and
norm[illegible]ls (hospital vol[illegible] [illegible]licon treated tubes. Eight of the pa-
tients [illegible]ell anemia had [illegible] multiple hospital admissions due to
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21 to 29 [illegible] *history of infe*[illegible] *the patients with sic*[illegible]ne-
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PMN leukocy[illegible]ls) [illegible]

2.5 μCi ($1^{14}C$)-D-glucose or 2.5 μCi ($6^{14}C$)-D-glucose. The second flask contained the same number of cells and isotope with the addition of latex polystyrene particles (0.81 μ). The amount of latex particles was 0.1 ml per 10^8 cells. The same experiment was performed using 2.5 μCi (^{14}C)-formate as an isotope. For the evaluation of electron acceptors on the conversion of ($1^{14}C$)-D-glucose to $^{14}CO_2$, leukocytes were incubated with 2.5 μC ($1^{14}C$)-D-glucose in the presence of 3×10^{-4} M methylene blue or 9×10^{-3} M sodium pyruvate. The chemical determination of the metabolic products has been previously described [4, 5, 6].

Oxygen consumption. Oxygen consumption was performed using a Clark type electrode (Oxygen Monitor System, Model 53). Polymorphonuclear leukocytes were suspended in KRB, pH 7.4, to a concentration of 0.5×10^8 cells per ml. Each cell of the oxygen monitor contained 3 ml of the suspension. Latex polystyrene particles 0.81 μ (0.15 ml) were injected through the side groove of the electrode. All experiments were performed at a temperature of 37 °C.

Nitroblue tetrazolium test (NBT). NBT test was performed by the method of BAEHNER and NATHAN [2]. The results were expressed as $OD/_{515}$ per 15 min per 2.5×10^6 cells.

Incubations in various media. To determine the influence of the SS serum on the phagocytic stimulation of PMN leukocytes, incubations were performed using serum from sickle cell patients and normal subjects as the incubation media. For controls, normal leukocytes were incubated in serum from sickle cell patients (III).

Phagocytic index. The phagocytic index was calculated using the method described by CLINE [3].

Results

Glucose utilization and activity of the hexose-monophosphate (HMP) shunt during phagocytosis. Glucose utilization of resting and phagocytizing PMN leukocytes from patients with sickle cell anemia (III) showed lower values than normal controls (table I). Leukocytes from patients with no history of infection and those with sickle cell trait utilize glucose normally. The direct oxidation of glucose via the hexose monophosphate shunt by resting leukocytes from all experimental groups is similar. No stimulation of the shunt occurred during phagocytosis in the sickle cell group with a history of infection.

Oxidation of formate. The relative measure of H_2O_2 production was demonstrated by the production of $^{14}CO_2$ from (^{14}C)-formate in resting and phagocytizing leukocytes [21]. PMN leukocytes from patients with sickle cell anemia (III) failed to show stimulation in the production of $^{14}CO_2$ as compared to the other groups (table I).

Oxygen consumption. Leukocytes from patients with sickle cell anemia (III) showed a decreased oxygen consumption at rest and a slight increase during phagocytosis. The difference in oxygen consumption be-

Table I. Effect of phagocytosis on glucose utilization, hexose monophosphate shunt, oxidation of ^{14}C-formate and oxygen consumption

		SS (HI)[1] mean ± SD (n = 8)	SS[2] mean ± SD (n = 5)	Sickle cell trait mean ± SD (n = 5)	Normal controls mean ± SD (n = 10)	p value[3]
Glucose utilization, µmoles/10^9 cells/h	resting	3.8 ± 1.9	5.5 ± 1.2	6.5 ± 0.8	5.8 ± 1.3	<0.02
	phagocytizing	4.2 ± 2.0	6.5 ± 1.1	6.9 ± 1.5	6.3 ± 1.2	<0.02
Hexose monophosphate shunt, %,	resting	2.2 ± 0.6	2.6 ± 1.1	2.4 ± 0.8	2.3 ± 0.6	<0.70
	phagocytizing	2.9 ± 0.9	12.1 ± 5.1	13.2 ± 2.1	13.2 ± 3.6	<0.001
Respiratory CO_2 from ^{14}C-formate, CPM/10^9 cells/h	resting	14,327 ± 2,109[4]	18,585 ± 399	18,966 ± 621	18,809 ± 772[4]	<0.001
	phagocytizing	14,773 ± 2,661[4]	37,422 ± 1,912	38,249 ± 1,139	37,986 ± 2,280[4]	<0.001
Oxygen consumption µl O_2/10^9 cells/h	resting	5.5 ± 2.8[4]	10.2 ± 2.8	9.2 ± 1.2	10.9 ± 2.7[4]	<0.02
	phagocytizing	8.4 ± 2.6[4]	27.6 ± 8.3	36.4 ± 5.4	40.3 ± 11.2[4]	<0.001

[1] SS (HI): Sickle cell anemia with history of infection.
[2] SS: Sickle cell anemia with no history of infection.
[3] p values are calculated for SS (HI) versus normal controls.
[4] n = 5.

tween these cells and the other control groups is significant (table I). No change in oxygen consumption was noted by addition of hemoglobin S to normal PMN leukocytes.

Effect of electron acceptors on the conversion of (^{14}C)-D-glucose to $^{14}CO_2$. Since the activity of the hexose monophosphate shunt was shown to be abnormally low in patients with sickle cell anemia (III), the operating condition of the shunt was further investigated. To exclude an enzymatic defect in the shunt, (^{14}C) D-glucose oxidation was studied in the presence of methylene blue and pyruvate [15]. As presented in table II, PMN leukocytes from patients with sickle cell anemia (III) showed an adequate stimulation of $^{14}CO_2$ production in all experimental groups. The total value of CO_2 produced by leukocytes from patients with sickle cell anemia is lower in comparison to the other groups.

NBT test. The reduction of NBT by leukocytes from patients with sickle cell anemia (III) was significantly lower during phagocytosis than the normal controls (table III). Although the leukocytes from patients with sickle cell anemia and no history of infection showed a significant reduction between resting and phagocytizing conditions the values were less significant than those for sickle cell trait and normal controls.

Effect of incubation medium on phagocytosis. Leukocytes from patients with sickle cell anemia (III) incubated in donor (SS) serum (5 experiments) and normal serum (3 experiments) showed no significant difference in glucose utilization and the hexose monophosphate shunt in resting and phagocytizing conditions. Leukocytes from normal individuals (3 experiments) incubated in serum from patients with sickle cell anemia (III) had normal stimulation of the hexose monophosphate shunt during phagocytosis.

Metabolic stimulation during phagocytosis by PMN leukocytes from splenectomized patients. Incubations were performed with PMN leukocytes from 3 splenectomized patients. The metabolic pattern of the leukocytes obtained from these patients corresponded to that of the normal control at rest and during phagocytosis.

Phagocytic index. The phagocytic index of PMN leukocytes from all experimental groups was calculated in order to correlate the metabolic response with particle uptake. The 8 patients with sickle cell anemia (III) included in this study had a phagocytic index of 71.6 ± 6.9 (± 1 SD)%, compared to the normal controls with 76.2 ± 7.2%. Leukocytes from 4 patients with sickle cell anemia not included in the metabolic study had a phagocytic index of less than 20%.

Table II. The effect of electron acceptors on the conversion of (1-^{14}C)-D-glucose to $^{14}CO_2$, CPM/10^{8} cells/30 min

Additive	Respiratory CO_2			
	SS (HI)[1] mean ± SD (n = 3)	SS[2] mean ± SD (n = 3)	Sickle cell trait mean ± SD (n = 3)	Normal controls mean ± SD (n = 5)
None	5,455 ± 649	10,377 ± 949	13,982 ± 1,592	14,20 ± 1,616
Methylene blue	69,720 ± 7,924	104,397 ± 11,923	124,145 ± 17,960	147,572 ± 18,392
Sodium pyruvate	17,017 ± 5,132	34,256 ± 4,696	36,818 ± 3,499	49,168 ± 7,688
p value[3]	<0.001	<0.001	<0.001	<0.001

[1] SS (HI) Sickle cell anemia with history of infection
[2] SS: Sickle cell anemia with no history of infection
[3] p values are calculated for no additive versus methylene blue and sodium pyruvate.

Table III Reduction of nitroblue tetrazolium, OD/$_{515}$ nm/2.5 10^{6} cells/15 min

	SS (HI)[1] mean ± SD (6)	SS[2] mean ± SD (5)	Sickle cell trait mean ± SD (5)	Normal controls mean ± SD (7)
Resting	0.08 ± 0.03	0.09 ± 0.03	0.10 ± 0.03	0.10 ± 0.03
Phagocytizing	0.11 ± 0.04	0.35 ± 0.15	0.41 ± 0.10	0.39 ± 0.11
p value[3]	<0.2	<0.05	<0.001	<0.001

[1] SS (HI). Sickle cell anemia with history of infection
[2] SS Sickle cell anemia with no history of infection
[3] p is the probability value for differences between resting and phagocytizing cells
Numbers in parentheses indicate number of experiments

Discussion

The present study of the phagocytic function of PMN leukocytes from patients with sickle cell anemia indicated that those patients with a history of repeated infection have defective leukocytes These leukocytes failed to

adequately stimulate respiration, hydrogen peroxide production and the activity of the HMP shunt during phagocytosis. The above metabolic abnormalities are shown to be associated with the failure of degranulation and defective intracellular killing of bacteria [1, 10, 11, 15] Further investigation of degranulation and killing effect of leukocytes from patients with sickle cell anemia could provide valuable information.

It has been demonstrated that the respiration of normal PMN leukocytes during phagocytosis is greatly stimulated due to an enhanced passage of glucose carbon through the HMP shunt [14, 15, 21] Thus, the integrity of this metabolic pathway is an important factor for the respiratory stimulation during phagocytosis Although leukocytes from patients with sickle cell anemia (HI) did not show stimulation of the HMP shunt during phagocytosis, they responded with adequate stimulation after the addition of methylene blue to the incubation medium. This suggests that failure to stimulate the HMP shunt is not due to the lack of enzymes responsible for the normal operation of this metabolic pathway [21]

It has been previously demonstrated [23] that leukocytes from patients with sickle cell anemia (HI) failed to show a significant reduction of tetrazolium dye during phagocytosis Identical results were obtained in our study A similar finding was demonstrated in leukocytes from patients with chronic granulomatous disease [1, 2, 11]

The role of serum opsonizing activity for the pneumococcus in patients with sickle cell anemia was clearly demonstrated by WINKELSTEIN and DRACHMAN [24] Their suggestion concerning functional asplenia in sickle cell anemia was later supported by PEARSON *et al* [18] Our study showed that leukocytes from splenectomized patients possess a normal phagocytic function accompanied by adequate metabolic stimulation

The suggestion of KAYE and HOOK [16] concerning the role of the hemolytic factor on the ability of the reticuloendothelial system to destroy bacteria was also considered during our experiments Hemoglobin S solutions did not affect to any extent either the activit of the HMP shunt or oxygen consumption during phagocytosis using leukocytes from normal individuals.

Regardless of the mechanism involved in this defect, an abnormality of the phagocytic function in leukocytes from patients with sickle cell anemia should be considered as an additional factor contributing to the increased susceptibility of these patients to infections. This defect could represent another granulocytopathy in addition to neutrophil dysfunction syndromes proposed by DOUGLAS *et al* [7]

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Acta Haemat 47 292–296 (1972)

Hemolytic Anemia and Pancytopenia in Glutathione Reductase Deficiency: Further Experience with Riboflavin[1]

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Abstract Three cases of hemolytic anemia and pancytopenia with glutathione reductase (GR) deficiency are described Since flavin adenine dinucleotide is the prosthetic group of GR, riboflavin was administered in order to increase the enzyme activity Despite the return of the GR activity to normal values, the hemolytic anemia and pancytopenia did not improve It is conceivable that our cases might represent a latent form of leukemia

Key Words
Glutathione reductase deficiency
Hemolytic anemia
Pancytopenia
Preleukemia
Riboflavin

Despite thorough studies of enzyme deficiencies in hemolytic anemia in recent years, no effective treatment could be established so far [3] Glutathione reductase (GR) is considered a key metabolic enzyme in erythrocytes Since flavin adenine-dinucleotide (FAD) is the prosthetic group of GR, it seems conceivable that this enzyme could be activated by flavin compounds [1, 9] We therefore tried to find out whether improvement of hemolytic anemia occurs following administration of riboflavin

Clinical Material and Methods

Our observations were based on 3 patients with pancytopenia and hemolytic anemia all revealed GR deficiency Before becoming symptomatic, 2 of our patients had been ingesting analgesics such as pyrazolone and salicylate for several

[1] Lecture presented in preliminary form at the 1st Meeting of the European Division of the International Society of Hematology Milano Italy Sept 1971

[2] Supported by Deutsche Forschungsgemeinschaft (Go 236/2)

Table I Relevant hematological findings

	Normal values	G. Ra., female, 22 y	D. Gu., male, 52 y	F. Ka., male, 67
Hb, g/100 ml	14–18	8.0	6.0	6.5
Hct, %	37–50	27	20	22
RBC, $\times 10^6$/mm³	4–5	2.6	1.8	2.4
Leucocytes, $\times 10^3$/mm³	5–8	3.0	2.8	2.6
Platelets, $\times 10^3$/mm³	150–300	11.0	8.0	9.0
Reticulocytes, %	0.8–1.5	1.5	6.0	4.0
RBC life-span, days	90–110	27.9	34.8	32.9
^{51}Cr RBC survival, t ½, days	25–35	14	17	12
Indirect bilirubin, mg%	0.6–0.8	2.46	1.8	2.2
Haptoglobin, mg%	25–170	0	30	44
Serum iron, µg%	85–145	238	250	160
GR activity, IU/10[11] RBC	11 ± 2			
before FAD		5.6	6.3	7.1
after FAD		10.8	12.9	12.7
GSH instability	<20	30	28	32
Heinz bodies	<20	26	30	18

months. A history of analgesic use in the third patient was questionable and unconfirmed. Physical examination of the patients showed no pathologic findings aside from striking paleness. Radiological studies, including a radioisotope scan of the spleen, proved negative.

Hematologic data are presented in table I and fig. 1. Serum iron and indirect bilirubin were increased and alpha$_2$-haptoglobin was reduced. Bone marrow aspiration revealed erythroid hyperplasia and toxic cytoplasmic granulation in neutrophils. The half life time (HLT) of the ^{51}Cr RBC was markedly reduced. Enzyme activities in the RBC were normal, except for diminished GR NADPH.

Test methods. Assays of the erythrocyte enzymes of glycolysis and the pentose phosphate shunt, including Heinz bodies and glutathione instability test, were carried out using the standard methods essentially described by BEUTLER *et al.* [5] and WALLER *et al.* [14]. Determination of the enzymes of the RBC were made on our 3 patients and several of their relatives. In GR electrophoresis, performed according to RENNER *et al.* [12] with cellogel strips, the isoenzyme band was absent prior to FAD incubation.

Results

The GR activity of hemolysates from these 3 patients revealed decreased values ranging from 5.5 to 6.5 IU/10[11] RBC. We found no en-

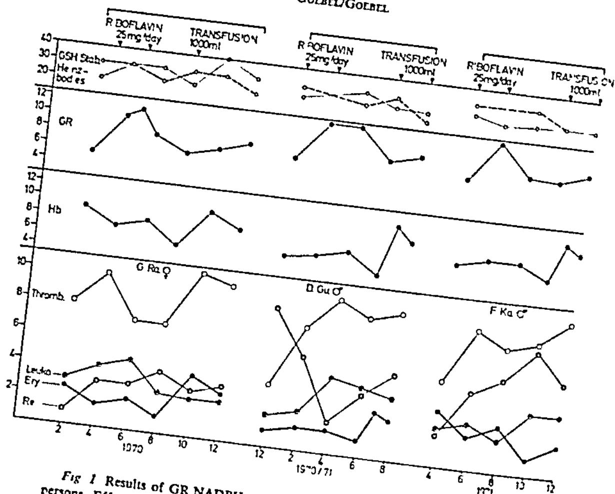


Fig. 1. Results of GR NADPH assay and other hematologic data of 3 affected persons. Effect of riboflavin (FAD) (intake for about 6 weeks) and transfusions. Glutathione (GSH) instability (Stab) •, Heinz bodies percentage of cells with more than 4, glutathione reductase (GR NADPH dependent) IU/10^{11} RBC; hemoglobin (Hb) g •, thrombocytes (× 10^5/mm^3), leukocytes (× 10^3/mm^3), reticulocytes •, erythrocytes (× 10^6/mm^3).

zyme deficiency in the examined relatives of GR deficient subjects. Since *in vitro* incubation of the hemolysate with FAD produced a clear increase of GR activity, each patient was given a daily dose of 25 mg of riboflavin for about 6 weeks. Details of the observed stimulation of GR activity are shown in figure 1. In GR electrophoresis, the isoenzyme II-band which had been absent before the test appeared in its full intensity after treatment with riboflavin. Despite the increase of GR to

normal values, neither the hemolytic anemia nor the pancytopenia improved. On the contrary, transfusions of fresh blood were necessary.

Discussion

Although it is evident that aplastic anemia can be improved by anabolic steroids [6] and splenectomy sometimes benefits the subject with severe pyruvate-kinase deficiency, both these treatments do not achieve a permanent remission. A causal therapy for enzymopenic anemia has not been described. GR deficiency has been considered the cause of hemolytic anemia [11]. Since GR is a flavoprotein with FAD as its coenzyme, administration of riboflavin might be expected to increase the GR activity [9] and thereby improve the anemia. In agreement with recent reports [1, 10, 11, 13], our results indicate a definite enhancement of GR activity in the RBC. After giving riboflavin, BEUTLER and SRIVASTAVA [2] achieved a return of FAD content in the red cells to normal levels. Nevertheless, the ^{51}Cr survival rate of the RBC remained unimproved so that the anemia persisted. In addition to the increase of GR activity, successful treatment with riboflavin in hemolytic or aplastic anemia has been claimed by other authors, who have nevertheless failed to submit details of their experimental or clinical findings [8].

Concerning the etiology of the GR deficiency, we assume the intake of analgesics to be a triggering factor in 2 of our cases. A genetically determined lack of GR appears unlikely in view of the negative findings in the relatives. In a rural tropical population FLATZ [7] has observed GR deficiency due to deficiency of vitamin B_2 in the diet. No indication of a deficiency of vitamin B complex was evident in our clinical findings, however. The results of our study suggest that factors other than GR deficiency are essentially involved in the etiology of hemolytic anemia and pancytopenia in our patients. Because conversion to the leukemias have been observed in hypoplastic anemia [4, 11], it is quite possible that our patients presented symptoms of preleukemia. The failure of the treatment with riboflavin could be explained on this basis [11, 13]. Our case report also supports the contention that we might be dealing with 2 different kinds of GR deficiency, a primary enzyme deficiency with hereditary nonspherocytic hemolytic anemia and a secondary lack of this enzyme associated with pancytopenia, which might possibly represent a latent form of leukemia.

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Acta Haemat. 47: 297–302 (1972)

The Effect of Certain Hormones on Platelet Aggregation *in vitro*

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Abstract. The effect of insulin, glucagon and triiodothyronin (T_3) on platelet aggregation was tested. It was shown that insulin and T_3 rendered the platelets hyperresponsive to all aggregating agents used. The 2nd phase of aggregation was especially potentiated. Glucagon had an inhibitory effect on platelet aggregation mainly on the 2nd wave of aggregation with adrenalin. Hypothyroid patients showed abnormal pattern of aggregation which was restored to normal after a week's treatment with T_3.

Key Words
Blood coagulation
Hormones
Platelet aggregation

In this paper, we report the effect of certain hormones on platelet aggregation *in vitro*, as measured by the turbidimetric method of Born [1] and O'Brien [7].

Material and Methods

The following hormones were given to normal subjects who had not received any drug for 2 weeks prior to start of the study.

Insulin cryst.: 0.2 units/kg body weight were administered i.v. to 12 volunteers. Blood samples were collected before and 25 min after administration, when the hormone had reached its maximum hypoglycaemic effect. At this time, the blood sugar was found to be <45 mg/100 ml.

Glucagon (Eli Lilly): 1 mg was given i.v. to 8 normal subjects. Blood was collected before and 10 min after injection of glucagon, when the blood sugar ranged from 100–140 mg/100 ml.

Triiodothyronin (T_3): 75 µg were given daily to 6 normal subjects for 5 consecutive days. Blood samples were collected before and at the end of the 5-day period.

Platelet aggregation tests were performed on 5 hyperthyroid patients. Five hypothyroids were also tested before and after treatment with T_4.

Aggregation study. Venous blood anticoagulated with 3.8% citrate (9:1) was centrifuged at 1 000 rpm for 15 min to obtain platelet rich plasma (PRP). The remaining blood was centrifuged at 3,500 rpm for 15 min to produce platelet poor plasma. The PRP was kept at room temperature and tested within 2 h. All glassware was siliconized. For the test aggregation 1 ml of PRP containing 300 000 platelets/mm³ was used. The platelet aggregation tests were performed according to the methods of BORN [1] and O'BRIEN [7] and with an EEL platelet aggregation meter, model 169. The transmission of light was recorded continuously with a desk type Servoscribe Potentiometric Recorder (Smith Industrie Limited) which was linked to the aggregometer. The PRP, divided into 1 ml samples, was placed in disposable polystyrene tubes, it was then transferred to a temperature-controlled chamber, and was stirred continuously. Each tube, before testing was prewarmed to 37 °C in a water bath for 3 min. Temperature was maintained at 37 °C and the speed of the stirrer kept arbitrarily at number 70 throughout the experiment. The sensitivity was set at 10 mV, the light transmission was recorded continuously at a chart speed of 600 mm/h.

The following aggregating agents were used: (a) adenosine diphosphate (ADP): the sodium salt of ADP (Sigma) was dissolved in barbitone buffer saline (BBS) pH 7.4 at a final concentration of 1 µM/ml PRP, (b) adrenalin was diluted in physiological saline and used at a final concentration of 5 µM/ml PRP, (c) collagen (connective tissue extract, CTE) was prepared with human tendon as described by HOVIG [4].

Results

Insulin. Insulin induced hypoglycaemia produced a marked increase in platelet clumping in citrated PRP with the 3 aggregating agents, as shown in figure 1. Adrenalin and ADP not only increased platelet aggregation in the PRP, but also promoted the onset of secondary aggregation, which is thought to be associated with the release of the platelet-intrinsic ADP [6]. The platelet aggregation pattern, obtained before and after *in vitro* addition of insulin into PRP, did not show any alteration.

Glucagon. Figure 1 shows that glucagon diminished platelet aggregation, especially when challenged with adrenalin, and retarded the initiation of the secondary clumping. The difference in the ADP- and CTE-inhibiting effect on aggregation was less obvious.

T_4. The aggregation pattern after administration of T_4 on PRP, compared with that before, is shown in figure 1. Both phases 1st and 2nd of ADP- and adrenalin induced aggregation were markedly increased, the aggregation was also markedly increased with CTE. Thus, the effect of T_4 was similar to that of insulin hypoglycaemia.

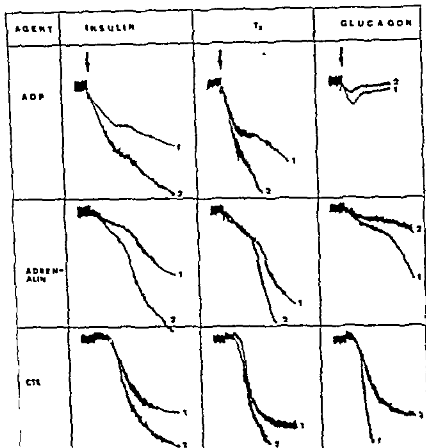


Fig. 1. Platelet aggregation with ADP, 1 μM final concentration (f.c.), adrenalin, 5 μM f.c., and CTE before (1) and after (2) administration of insulin, T_3 and glucagon.

Normal platelet aggregation with ADP, adrenalin and CTE was seen in hyperthyroid patients. However, platelets from myxoedematous patients responded very weakly in the aggregation tests with ADP, adrenalin or CTE. The aggregating tracings were abnormal and no disaggregation was observed with ADP. The pattern was similar to that seen in thrombocytopathia as regards the adrenalin- and CTE-induced aggregation. This disturbed platelet function was completely restored to normal after a week's treatment with T_3 (fig. 2).

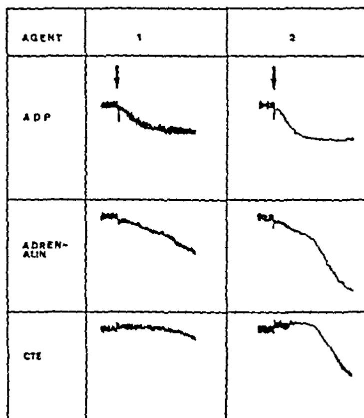


Fig 2 Platelet aggregation on an hypothyroid patient before (1) and after (2) *therapy with* T_3

Discussion

Insulin-induced hypoglycaemia caused enhancement of platelet aggregation This was probably not a direct effect of insulin on platelets, since *in vitro* addition of the agent to PRP did not show any change in the normal aggregation patterns It is most likely that the effect of insulin on platelets was an indirect one During insulin-induced hypoglycaemia, the plasma adrenalin rose from <0 06 to 1 8 μg/ml [3] The increased concentration of adrenalin in the plasma might accelerate the onset of the 2nd phase of platelet aggregation [8] Part of the secreted adrenalin and noradrenalin is taken up by blood platelets [2] through an active transport mechanism [10] It has been estimated that 1 ml PRP contains *1–3 ng catecholamines, so that platelet aggregation can be potentiated*

by its own catecholamines, if they are released. It is, therefore, reasonable to surmise that the enhanced platelet aggregation during hypoglycaemia is due to an increased amount of platelet catecholamines, released during the 2nd phase of aggregation

We also found that T_3 enhanced platelet aggregation in normal subjects in a similar pattern as in the case of insulin This is difficult to explain It is known that T_3 promotes various metabolic actions of adrenalin, and this could account for the increased platelet aggregation We showed that platelet aggregation in hypothyroid patients was abnormal (decreased), it was restored to normal following treatment with T_3

Glucagon, on the other hand, given i v, moderately inhibited platelet aggregation A great inhibiting effect was noticed when PRP was challenged with adrenalin The mode of action of glucagon on platelet aggregation is not clear As ROBISON *et al* [9] have suggested, glucagon increases the effectiveness of adenyl cyclase, which in turn increases the concentration of cyclic 3 5 -AMP in the cells Whether the glucagon induced inhibition of platelet aggregation is due to the increased concentration of cyclic 3 5 -AMP remains speculative The inhibiting effect of cyclic 3 5 -AMP on the ADP- and adrenalin induced aggregation is well known [5]

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Acta Haemat. 47: 303–310 (1972)

Evaluation of Glucose-6-Phosphate Dehydrogenase in Single Erythrocytes in Human Blood Smears[1]

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Abstract. A cytochemical method has been described for the specific and direct staining of glucose-6-phosphate dehydrogenase (G-6-PD) in red blood cells (RBC) by using the tetrazolium salt Nitro-BT (NBT). This method allows a semiquantitative estimation of G-6-PD activity in single cells. The results of such estimations were found to be correlated with spectrophotometric determination of G-6-PD activity in RBC lysates. The activity of G-6-PD was shown to decline with aging of the cell. In cases of severe G-6-PD deficiency, where the spectrophotometric assay revealed no activity, staining of 'young' RBC showed appreciable activity, while the 'old' cells showed no activity.

Key Words
Cytochemistry
Erythrocyte enzymes
G-6-PD staining
G-6-PD deficiency

Several cytochemical methods have been described for the detection of glucose-6-phosphate dehydrogenase (G-6-PD) activity in erythrocytes [1]. However, these methods are indirect and are based on the reduction of methemoglobin into oxyhemoglobin, which is related to the G-6-PD activity of the red blood cell (RBC), in the presence of an artificial electron carrier such as methylene blue.

In this work, we present a direct and specific staining for G-6-PD activity in the RBC by using the tetrazolium salt Nitro-BT (NBT) as an electron acceptor [2]. This method has been used for semi-quantitative cytochemical estimation of G-6-PD in human erythrocytes by counting the formazan granules formed as a result of the reaction. The evaluation of enzymatic activity was made on regular blood smears and on smears of

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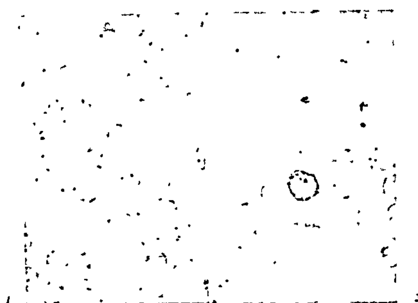


Fig. 1 Staining for G-6-PD activity in erythrocytes of normal peripheral RBC.

Fig. 2 Blood smear from a case of severe G-6-PD deficiency stained for enzymatic activity. In the center a single cell (young) with strong G-6-PD activity

tial. Focusing the microscope and selecting the appropriate field was done by using a low magnification phase-contrast objective (16 ×). For the estimation of activity, an oil immersion phase-contrast objective (100 ×) was used.

The enzymatic activity was estimated by grading the cells according to the number of formazan granules present in each cell: no granules 0, 1–2 granules +1, 3–6 granules +2, 7–10 granules +3, 10 granules +4. In each case 200 to 500 erythrocytes were examined. The sum of the grading for 100 RBC constitutes the score as used in this study.

Assay of G-6-PD in venous blood. G-6-PD activity was determined by a screening method [4] in all cases and by the spectrophotometric assay [5] in some of the blood samples taken.

Glucose determination in erythrocytes [6]: the value of the glucose/g hemoglobin was determined in erythrocytes separated into "young" and "old" cells from 6 healthy subjects.

As a control for specificity of the staining method, blood smears and smears prepared from "young" and "old" cells from 12 normal subjects were incubated with the incubation solution lacking the specific substrate glucose-6-phosphate.

The influence of the glucose content in erythrocytes on G-6-PD activity. Venous blood was collected in a sterile heparinized tube, kept at 4 °C, and during one week daily smears of "young" cells were stained for G-6-PD with and without glucose-6-

erythrocytes separated according to their age, from peripheral blood of normal subjects and from patients suffering from G-6-PD deficiency

Materials and Methods

For semiquantitative cytochemical determination of G 6-PD activity in erythrocytes, thin smears of peripheral blood were used Blood donors were 29 healthy subjects (18 females and 11 males) and 23 patients suffering from G-6-PD deficiency (7 females and 16 males) all having the Gd Mediterranean G 6-PD variant In addition blood smears were taken from 11 healthy subjects (5 females and 6 males) and from 8 patients with G-6-PD deficiency (2 females and 6 males) whose erythrocytes were separated according to their age

To separate the erythrocytes into age groups, capillary or venous blood was taken in heparinized capillaries used for microhematocrit. Fractionation into age groups was done using phthalate esters [3] For separation of young erythrocytes, phthalate esters having densities of 1 098, 1 094 and 1 090 g/ml were used while for separation of 'old' erythrocytes, phthalate esters having densities of 1 106 or 1 110 g/ml were employed

For the preparation of the blood smears, the fractionated erythrocytes were diluted 1 1 with autologous plasma.

Cytochemical staining The presence of G 6-PD in the erythrocytes was demonstrated by exposing the blood smears to a buffered incubation solution containing NBT and the specific substrate D glucose-6-phosphate The G 6-PD reaction is coupled to NBT as the terminal H^+ acceptor by means of tetrazolium reductase (diaphorase) which causes the transfer of H^+ from $NADPH_2$ to NBT The latter, on accepting hydrogen ion is changed into insoluble formazan which is precipitated in the form of small grains on the cells

The incubation solution contains equal volumes of NBT 1 mg/ml D glucose-6-phosphate disodium salt 0.2 M and NADP 1 mg/ml of Sorensen s phosphate buffer M/15 at pH 7 4

In preparing and staining the blood smears, great care should be taken to avoid cell damage or destruction since staining is done on unfixed cells The microscope slide must be very clean and the smears should be made thin enough so that individual cells can be easily distinguished. The fresh smears are dried in air for 30-60 min and are stained by adding one or two drops of the incubation solution, subsequently they are covered with coverslips and are placed in a humid Petri dish at 37 °C for 2 h. The incubation solution should not be in excess and the coverslips should be firmly placed on the slide Excess solution will cause the cells to float After incubation the coverslips are carefully removed and the smears are dried in air and fixed in formalin fumes at 25 °C for 7 min. They are then washed under running tap-water for 5 min and stained for 10 min with 0 1% neutral red Several blood smears should be made from each sample

The smears should be checked and counted in areas where the cells are completely separated and maintain structural integrity Because of the very slight contrast between the RBC and the glass the use of phase-contrast microscopy is essen

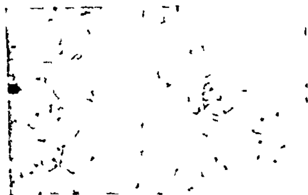


Fig 4 Enzymatic staining of normal 'young' erythrocytes

Normal 'young' cells show a very intense enzymatic staining as compared with unfractionated normal cells or with cells separated as 'old'. Most 'young' cells were graded as +3 or +4 and practically all the cells showed enzymatic activity (fig 4). In old cells on the other hand the cells that did show G-6-PD activity were mostly graded as +1. This difference between 'young' and 'old' cells is more pronounced in cases of G-6-PD deficiency (fig 5). The cells separated as 'old' showed no G-6-PD activity at all, while in smears prepared from 'young' cells the score of the enzymatic activity was higher as compared with unfractionated erythrocytes.

The G-6-PD activity in unfractionated 'young' and 'old' cells determined by the spectrophotometric assay and the cytochemical evaluation is shown in figures 5 and 6. The cytochemical assay in normal cases revealed activity distribution similar to the spectrophotometric assay. In cases of G-6-PD deficiency the cytochemical staining demonstrated essentially the same activity distribution as was found in normal RBC. In deficient cases the 'old' cells showed practically no activity at all, while the 'young' cells were active and their scores varied from 20 to 150. On the other hand the score of unfractionated blood smears was 2–50 while normal unfractionated blood smears gave a score of 130–260.

The control values for normal unfractionated blood stained in the absence of glucose-6-phosphate showed little activity as evidenced by a few scattered granules in a small percentage of the cells (score of 0–14) (fig 5). Control values for normal 'young' cells showed a slightly higher activity while normal 'old' cells showed no activity at all in the absence of glucose-6-phosphate.

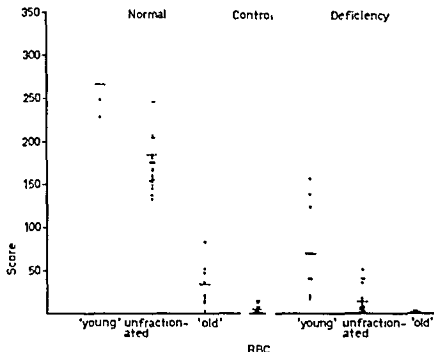


Fig 5 Cytochemical evaluation of G 6-PD activity in 'young', 'old' and unfractionated RBC in smears of normal subjects and in cases of G 6 PD deficiency Control normal unfractionated blood stained in the absence of glucose 6-phosphate

The glucose content of 'young' erythrocytes was 20–60% higher as compared to 'old' erythrocytes

Venous blood which was kept at 4 °C revealed a decrease in the sugar content and no change in the spectrophotometric and cytochemical determination of G-6-PD However, in staining of the 'young' cells in the absence of the glucose-6-phosphate, practically no enzymatic activity could be detected after 7 days at 4 °C

Discussion

The staining method described above stains the cells directly for G-6-PD activity by coupling the G-6-PD reaction to NBT as the terminal H' acceptor, in this way, the activity in individual cells can be estimated Previously described techniques for G 6-PD staining require prior oxidation of hemoglobin into methemoglobin by nitrite treatment and then coupling the G-6-PD reaction to methemoglobin reduction by introducing a dye which links $NADPH_2$ oxidation to methemoglobin reduction The

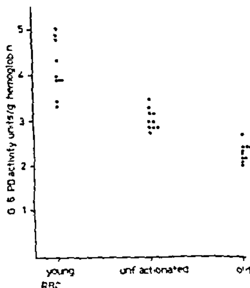


Fig 6 Comparison between G-6-PD activity of 'young', 'old' and unfractionated erythrocytes from normal donors. Enzymatic activity was determined spectrophotometrically

cells are then stained after the elution of the residual methemoglobin [1] or by the reduction of MTT tetrazolium into insoluble formazan by oxyhemoglobin [7]

In either case the staining is indirect and involves several additional enzymatic steps and can show either active or inactive cells, but cannot distinguish between very low and very high activity in the cells

In the present method the staining is directly related to the G-6-PD reaction product NADPH, and can be quantitated by counting the formazan granules formed

The fact that the G-6-PD activity declines in the cells with aging was shown here at the single cell level. It appears that the low activity observed in the absence of added substrate in 'young' and unfractionated erythrocytes, can be ascribed to production of glucose-6-phosphate in small amounts by the unfixed cells. In 'old' cells, on the other hand, the low hexokinase, G-6-PD and glucose levels present, will result in an insignificant reaction due to endogenous supply of glucose-6-phosphate

The cytochemical estimation was correlated with the spectrophotometric assay and in cases of severe deficiency where the enzyme activity

in RBC could not be detected by the spectrophotometric assay, low G-6-PD activity could be identified by the cytochemical procedure.

Attempts to demonstrate the X chromosome mosaicism were made by using indirect G-6-PD staining of the cells and looking at the percentage of active and inactive cells in cases of heterozygote females [1, 7]. Using the methemoglobin elution technique, 2 RBC populations were found for cases of mild G-6-PD deficiency in males [8]. It was shown here that in cases of severe G-6-PD deficiency (males), where every cell is mutated, up to 40% of the cells can show G-6-PD activity and the percentage of active cells will depend, probably, on the percentage of 'young' cells in the particular donor. On the other hand, in normal cases, the 'old' cells have a rather low G-6-PD activity and a large fraction of these cells can be inactive. These combined findings lead to the conclusion that mutated or deficient cells can be active and normal cells can be inactive. By using this cytochemical NBT method, the number of inactive cells cannot, therefore, be considered a dependable criterion for determination of X chromosome mosaicism

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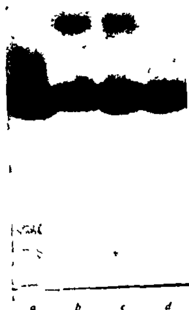


Fig 1 Starch gel electrophoresis pH 8.1 of (a) case No 9 (b) case No 7 (c) case No 5 and (d) normal subject

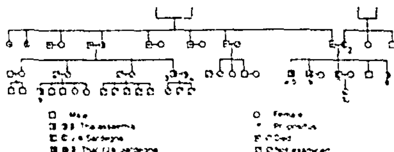


Fig 2 Family tree

The tryptic fingerprint (fig 5) showed that the normal α-Tp VI was missing while a new positive tyrosine and histidine peptide was located immediately below the normal β Tp IX. As can be seen from table II this had one less histidine and one more aspartic acid residue than the normal

Material and Methods

The propositus and 2[illegible] members of his family were examined. Routine blood studies and starch gel electrophoresis (buffer: tris EDTA borate at pH 8.1 for the gel, pH 9.0 for the vessels) [7, 18] were run on each subject. The technique of Ricco *et al.* [14] was used to evaluate the fractions. Haemoglobin fractions were chromatographically separated on Sephadex DEAE A 50 (2.5 cm × 100 cm) anion exchange column, using a pH gradient buffered from 8.1 (tris HCl 0.05 M) to 7.0 [9]. After vacuum concentration, α- and β-chains were separated by incubation with parachloromercuribenzoate (PCMB) and examined by starch gel electrophoresis at pH 8.3 [15]. The abnormal fraction was converted to globin by cold precipitation with HCl-acetone, digested with trypsin and fingerprinted [16]. The α-T peptide was eluted from several preparative fingerprints, digested with chymotrypsin and refingerprinted [16]. Amino acid analysis was carried out on an Omnia amino acid analyser after elution and hydrolysis with HCl 6 N at 110 °C for 18 h.

In the propositus, α- and β-chain synthesis was studied on peripheral blood incubated with ^{14}C leucine for 120 min, according to Kan *et al.* [9]. The chains were separated by means of the method of Clegg *et al.* [4]. The oxyhaemoglobin dissociation curve was obtained by equilibrating various samples of blood at 37 °C with oxygen-nitrogen mixtures at various P_{O_2} and by measuring their blood O_2 content by the manometric method of Van Slyke and Neill [2[illegible]].

O_2 capacity values were determined by multiplying the haemoglobin content in the cyanmethaemoglobin by 1.36. The results were corrected for the measured pH (using a pH Meter) by means of the equation of Dill *et al.* [5]. 2,3-DPG was determined with the method of Krimsky [1[illegible]].

Results

The propositus was a 28-year-old male from Cagliari (Sardinia) with a history of slight liver and spleen enlargement and fluctuating scleral subicterus of some years' standing. His blood picture, together with those of selected members of his family, is illustrated in table I.

The propositus showed 23.8% Hb J and 5.3[illegible]% Hb A; the latter was composed of 2 fractions with normal (4.2[illegible]%) and fast (1.1[illegible]%) migration rates, respectively (fig. 1).

The genealogical tree for this family is given in figure 2. Of the 2[illegible] subjects examined, 3 were carriers of Hb J Sardegna, 5 displayed the thalassaemia trait, and 2 presented Hb J Sardegna/thalassaemia double heterozygosity. The presence of Hb J was both clinically and haematologically silent, while the 2 thalassaemic groups presented similar blood and clinical pictures. No percentage Hb J differences were apparent.

In the propositus, resin column chromatography was employed to isolate the Hb J fraction (fig. 3). Dissociation with PCMB revealed the presence of fast α-chains in the purified fraction (fig. 4).

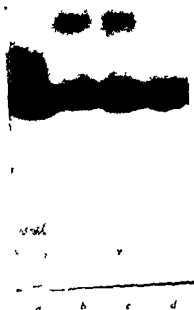


Fig. 1. Starch gel electrophoresis pH 8.1 of (a) case No. 9 (b) case No. 7 (c) case No. 5 and (d) normal subject

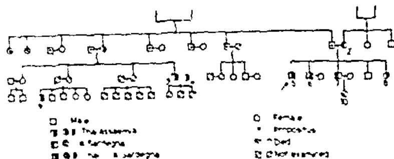


Fig. 2. Family tree

The tryptic fingerprint (fig. 5) showed that the normal α-Tp VI was missing, while a new positive tyrosine and histidine peptide was located immediately below the normal β Tp IX. As can be seen from table II, this had one less histidine and one more aspartic acid residue than the normal

Table I Haematological and haemoglobin electrophoretic findings in the affected members of the propositus's family

Case No	Hb, g%	RBC $\times 10^6$/μl	PCV, %	MCV, μm^3	MCHC, %	Retics, %	Morphology[1]			Electrophoresis				Diagnosis
							A	P	T	$A_2^A+A_2^J$	F	A	J	
1	10.9	4.7	33	70	33	3.0	++	+	+	3.9	1.0	95.1	–	β-thalassaemia
2	15.4	5.0	47	94	32	0.7	normal			2.8	0.4	80.5	16.3	J Sardegna
3	13.0	4.6	38	82	34	1.8	+	+	+	4.5	0.6	94.9	–	β-thalassaemia
4	11.2	4.4	35	79	32	2.1	++	++	+	4.1	0.7	95.2	–	β-thalassaemia
5	14.0	5.2	42	80	33	1.5	++	+	++	5.3	0.7	70.2	23.8	β thal /J Sardegna
6	13.8	5.0	40	80	34	1.2	+	+	+	5.3	0.9	72.6	21.2	β thal /J Sardegna
7	15.2	5.0	48	96	32	0.5	normal			2.7	0.5	76.5	20.3	J Sardegna
8	11.3	4.5	38	84	30	2.2	++	+	+	5.7	1.2	93.1	–	β-thalassaemia
9	11.7	4.7	39	83	30	1.6	+	++	+	4.3	0.5	95.2	–	β-thalassaemia
10	14.8	4.6	44	95	31	0.6	normal			3.1	0.4	77.4	19.1	J Sardegna

[1] A = Anisocytosis, P = poikilocytosis, T = target cells

Table II Amino acid composition of normal and abnormal α Tp VI

Residues of amino acids	Hb A	Hb J
Aspartic acid	*1.0*	*2.3*
Threonine	0.9	1.0
Serine	2.1	2.1
Glutamic acid	1.0	1.2
Proline	1.1	1.2
Glycine	1.2	0.9
Alanine	1.1	1.2
Valine	1.0	0.9
Leucine	1.0	1.0
Tyrosine	1.0	0.9
Phenylalanine	1.8	1.7
Histidine	*2.2*	*1.1*
Lysine	1.0	1.2

Amino acid sequence of the tryptic peptide α Tp VI of Hb A and Hb J Sardegna

Hb A Thr Tyr Phe Pro His Phe Asp Leu Ser *HIS* Gly Ser Ala Glu Val Lys
Hb J Thr Tyr Phe Pro His Phe Asp Leu Ser *ASP* Gly Ser Ala Glu Val Lys
41 42↑[1] 43 44 45 46↑ 47 48 49 50 51 52 53 54 55 56

[1] The arrows indicate points of chymotryptic cleavage

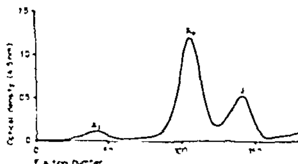


Fig. 3 DEAE cellulose chromatography of Hb J Sardegna

α Tp VI. In this connection, however, it should be noted that glutamine and asparagine are converted into glutamic and aspartic acid respectively by acid hydrolysis prior to analysis. The normal α-Tp VI has histidines in position 45 and 50

Table I Haematological and haemoglobin electrophoretic findings in the affected members of the propositus's family

Case No	Hb g%	RBC $\times 10^6/\mu l$	PCV, %	MCV, μm^3	MCHC, %	Retics, %	Morphology[1]			Electrophoresis				Diagnosis
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1	10.9	4.7	33	70	33	3.0	++	+	+	3.9	1.0	95.1	–	β thalassaemia
2	15.4	5.0	47	94	32	0.7	normal			2.8	0.4	80.5	16.3	J Sardegna
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4	11.2	4.4	35	79	32	2.1	++	++	+	4.1	0.7	95.2	–	β-thalassaemia
5	14.0	5.2	42	80	33	1.5	++	+	++	5.3	0.7	70.2	23.8	β-thal /J Sardegna
6	13.8	5.0	40	80	34	1.2	+	+	+	5.3	0.9	72.6	21.2	β-thal /J Sardegna
7	15.2	5.0	48	96	32	0.5	normal			2.7	0.5	76.5	20.3	J Sardegna
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9	11.7	4.7	39	83	30	1.6	+	++	+	4.3	0.5	95.2	–	β-thalassaemia
10	14.8	4.6	44	95	31	0.6	normal			3.1	0.4	77.4	19.1	J Sardegna

[1] A = Anisocytosis, P = poikilocytosis, T = target cells

Table II Am no acid composition of normal and abnormal α Tp VI

Residues of am no acids	Hb A	Hb J
Aspartic acid	*1.0*	*2.3*
Threon ne	0 9	1 0
Serine	2.1	2.1
Glutamic acid	1 0	1.2
Prol ne	1 1	1.2
Glycine	1.2	0 9
Alan ne	1 1	1.2
Val ne	1 0	0 9
Leucine	1 0	1 0
Tyrosine	1 0	0 9
Phenylalanine	1 8	1 7
Histid ne	*2.2*	*1 1*
Lysine	1 0	1.2

Am no acid sequence of the tryptic peptide α Tp VI of Hb A and Hb J Sardegna

Hb A Thr Tyr Phe Pro His Phe Asp Leu Ser *HIS* Gly Ser Ala Glu Val Lys
Hb J Thr Tyr Phe Pro His Phe Asp Leu Ser *ASP* Gly Ser Ala Glu Val Lys
41 42↑[1] 43 44 45 46↑ 47 48 49 50 51 52 53 54 55 56

[1] The arrows indicate points of chymotryptic cleavage

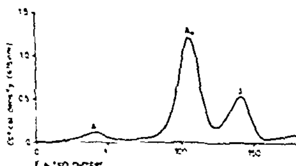


Fig. 3 DEAE cellulose chromatography of Hb J Sardegna.

α Tp VI. In this connection, however, it should be noted that glutamine and asparagine are converted into glutamic and aspartic acid, respectively, by acid hydrolysis prior to analysis. The normal α-Tp VI has histidines in position 45 and 50



Fig. 4. Starch gel electrophoresis pH 8.3 of (a) Hb A, (b) α- and β-chains of Hb A, (c) α- and β-chains of partially purified Hb J, and (d) Hb J.

It was thus necessary to determine which of these had been replaced and whether the newcomer was an aspartic acid or an asparagine residue. Comparison of the normal and pathological chymotryptic fingerprints for the α-VI peptide showed that the usual positive α 47-56 fragment (A, fig. 6) had been replaced by a negative fragment in a more anodic position (J, fig. 6). The mutation, therefore, involved the substitution of histidine 50 by an aspartic acid and was typical for Hb J Sardegna.

In the propositus, the β/α A+α J ratio was 0.67 (fig. 7); this falls within our range of values for the thalassaemia trait. α-J-chain radioactivity was 18% of the total for the α-chains.

The oxygen dissociation curve was normal, with P_{50} = 25 (our normal laboratory value is 26 ± 1); the 2.3 DPG value, expressed in μmoles/ml red cells, was 4.91 (our normal laboratory value is 4.95 ± 0.6).

Discussion

The presence of Hb J Sardegna did not appear to give rise to appreciable changes in the haematological picture. Heterozygote carriers displayed normal values, while subjects with double Hb J β-thalassaemia heterozygosis presented clinical and haematological signs typical of β-thalassaemia heterozygotes. The oxygen dissociation curve indicated that the substitution of aspartic acid for histidine had not disturbed oxygen affinity.

In the propositus, the relative rate of synthesis of the α- and β-chains was similar to that observed by us in heterozygote β-thalassaemia. The

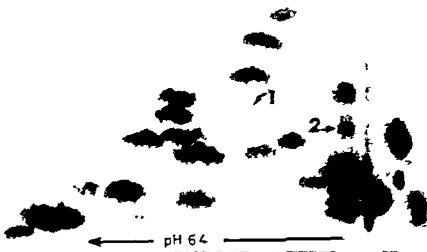


Fig. 5 Fingerprint of Hb J Sardegna. α-Tp VI of Hb A is missing (1); α-Tp VI of Hb J Sardegna (2)

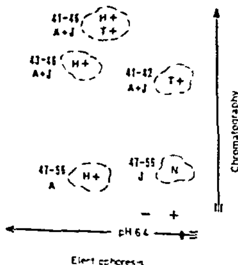


Fig. 6 Fingerprint of the chymotryptic peptides of α-Tp VI of Hb A and Hb J Sardegna. H = Histidine. T = Tyrosine. N = ninhydrin.

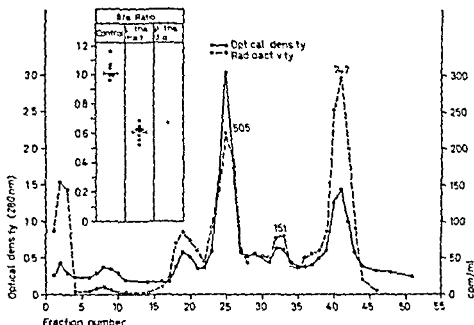


Fig 7 Column chromatography on CMC in 8 M urea of globin from a haemolysate prepared after incubation of reticulocytes of the propositus with ^{14}C leucine for 120 min Total incorporation of radioactivity into β, α J and α A chains is indicated near the peaks Insert shows the ratio of specific activity β/α (expressed as cpm/ml/OD 280) in 6 normal subjects, in 9 β thalassaemia carriers and in the propositus

ratio between α J and α A chain radioactivity corresponded to the ratio between the Hb J and Hb A fractions in the peripheral blood

The interaction of thalassaemia and a structural gene variant is most clearly evident in the combination of β-thalassaemia with β-variant, or α-thalassaemia with α-variant In these instances, there is a considerable increase in the percentage of abnormal haemoglobin, examples include β-thalassaemia Hb S [2] and α-thalassaemia/Hb Q [6] Reduced abnormal haemoglobin synthesis, on the other hand, is evident in α-thalassaemia/β-variant associations, such as α-thalassaemia/Hb S [25] or α-thalassaemia Hb E [22]

The combination of β-thalassaemia with α-variants, however, displays no form of interaction In our series, for example, an Hb J percent range of 16 3–23 8 covered the data for the simple heterozygosis for Hb J and its association with β-thalassaemia

It has been observed that, while most α-variants represent about 25% of the total haemoglobin content, this figure is often doubled in the case

of β variants. Lehmann and Carrell [11] have advanced the view that α-chain synthesis is regulated by 2 pairs of genes. They suggested that in the case of heterozygotes for an α variant only one of the 4 genes is abnormal and that this may be the reason for the low percent of abnormal haemoglobin fraction.

Our α A and α J-chain synthesis data show that the latter forms 18% of the α-chain total; this finding is in line with the views of Lehmann and Carrell [11]. A recent paper by Brimhall *et al.* [3] described a Hungarian family in which the normal Hb A of one subject was flanked by two α variants, showing that regulation of α-chain synthesis by 2 pairs of genes is certainly possible. The report on a subject homozygote for Hb J α Tongariki [1], however, means that the question is still open unless it is held that the number of structural loci for α-chains is not consistent in different populations.

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Acta Haem. 47: 321-325 (1972)

Immunization of Guinea Pigs against Leukemia by Scarification of Skin with Leukemic Cell Extracts[1]

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Abstract. L2C leukemic cell suspensions consistently induce a generalized and fatal stem-cell leukemia, when inoculated subcutaneously or intraperitoneally into young, adult 'strain 2' or F_1 hybrid guinea pigs. In experiments here reported 'strain 2' and F_1 hybrid guinea pigs were inoculated by superficial skin scarification with small doses of diluted L2C leukemic cell suspensions. When about 40 to 50 days later such animals were reinoculated subcutaneously with massive doses (0.5 ml each) of leukemic cell suspensions of 10^{-3} concentration, 30 of 31 guinea pigs tested were found to be immune to the challenging inoculation. The same dose induced a generalized leukemia in all 45 untreated 'strain 2' and in 29 of 30 F_1 untreated hybrid guinea pigs.

Key Words
Immunity against leukemia
Leukemia in guinea pigs

We have reported in our previous studies [3] that 'strain 2' and F_1 hybrid guinea pigs could be immunized against L2C leukemia by intradermal inoculation of very small doses of leukemic cell suspensions. The small intradermal tumors which resulted from such inoculations, regressed spontaneously in about 50% of the animals. Most of the guinea pigs in which the intradermal tumors regressed were solidly immunized against reinoculation of L2C leukemic cells by any route [3, 4].

In our current studies we have now observed that immunity against leukemia can also be induced in guinea pigs by superficial scarification of the skin with leukemic cell suspensions, instead of inoculating such leukemic cell extracts intradermally.

Materials and Methods

L2C leukemia. The L2C leukemia strain, employed in this study, originated some 18 years ago as a spontaneous leukemia in one of the 'strain 2' guinea pigs [1] and has

[1] Aided in part by grants from the Damon Runyon Memorial Fund, the American Cancer Society and the Chemotherapy Foundation of New York.

been maintained since that time by serial passage, presumably by cell graft, in animals of the 'strain 2' inbred line [5, 6]. When L2C leukemic cell suspensions are inoculated subcutaneously or intraperitoneally into 'strain 2' or F_1 hybrid guinea pigs, they consistently induce a rapidly progressing and uniformly fatal, generalized stem-cell leukemia.

Animals. Our experiments were carried out on 'strain 2' guinea pigs bred in our laboratory by brother-to-sister mating, and also on F_1 hybrids, born in our laboratory to Hartley females and 'strain 2' males.

Preparation of leukemic cell suspensions. A guinea pig with advanced leukemia was sacrificed by ether inhalation. A fragment of the subcutaneous leukemic tumor from the site of inoculation, and also a small fragment of spleen, and of the mesenteric tumor, were removed aseptically, weighed, cut with scissors, and ground in a mortar, sterile physiological saline solution being added to obtain a cell suspension of 10^{0} concentration; the cell extract was then passed through a sterile voile cloth. Serial dilutions of desired concentration were prepared from the original cell suspension, and used immediately for inoculation [4, 5].

Scarification of skin. The scarification was performed in a similar manner to that employed in the application of smallpox vaccine to the skin. A few drops of a leukemic cell suspension of desired concentration were placed on the skin of the flank of a guinea pig on an area on which the hair had been closely clipped with an electric shaver. A sharp, sterile, 26-gauge needle was then used to scarify the skin on an area of about one square inch, introducing thereby the leukemic cell extract into the superficial layers of the skin. The scarification was performed gently so as not to pierce the skin too deeply with the needle during this procedure. Each of the scarified animals was then placed on an individual small table for 1–2 hours to allow the scarified skin to dry. No dressing was applied. The animals were then returned to their cages.

Results

Among the 44 guinea pigs inoculated by skin scarification, 5 developed leukemia, particularly in those groups in which relatively higher concentrations of leukemic cells were applied (table I). In 4 instances, small intradermal tumors developed after 19–24 days at the site of scarification; they regressed spontaneously, 7–22 days later, without trace. In the majority of animals, however, the scarified areas healed within 8–12 days, with no local infection discernible and no development of a local skin tumor.

Resistance of Guinea Pigs, Following Skin Scarification, to a Challenging Reinoculation of Leukemic Cells

31 guinea pigs, which had their skin scarified with leukemic cell extracts, were submitted 35–59 days later to a challenging subcutaneous reinoculation of massive doses (0.5 ml each) of leukemic cell suspensions of 10^{-2} concentra-

Table I. Result of scarification of skin in 'strain 2' and F_1 hybrid guinea pigs with leukemic cell suspensions

Leukemic cell concentration	Number of guinea pigs scarified	Number developed transient ‚d tumors	Number developed generalized leukemia	Average time of leukemia development days	Leukemia incidence %
10^{1}	3	0	0		
10^{2}	4	0	1	45	25
10^{3}	22	1	4	42	18
10^{4}	15	3	0	0	0
Total	44	4	5		11

Table II. Resistance of guinea pigs, following skin scarification, to challenging reinoculation of leukemic cells

Route of challenging inoculation	Average time interval after scarification days	Number of guinea pigs inoculated	Number developed leukemia	Average time of leukemia development days	Leukemia incidence %
Subcutaneous[1]	47	31	1	56	
Intradermal[2]	33	5	3	25	
Total		36	4		11

[1] Subcutaneous challenging inoculation consisted of 0.5 ml of a leukemic cell suspension of 10^{2} concentration.

[2] The intradermal challenge consisted of 0.2 ml of a leukemic cell suspension of a 10^{2} concentration.

tion. All, except one, remained in good health during an observation period, which, for most of them, has extended for a period of over 6 months (table II). In a control experiment, 45 'strain 2' and 30 F_1 hybrid normal, untreated, guinea pigs were inoculated subcutaneously (0.5 ml each) with leukemic cell suspensions of 10^{2} concentration; as a result, within 3 weeks, all 45 'strain 2' guinea pigs and 29 out of 30 F_1 hybrids, developed, and died from, a generalized, stem-cell leukemia.

been maintained since that time by serial passage, presumably by cell graft, in animals of the 'strain 2' inbred line [5, 6]. When L2C leukemic cell suspensions are inoculated subcutaneously or intraperitoneally into 'strain 2' or F_1 hybrid guinea pigs, they consistently induce a rapidly progressing and uniformly fatal, generalized stem-cell leukemia.

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A certain amount of time is required for the immunity to develop after scarification. For that reason, we have delayed the challenging, subcutaneous reinoculations of massive doses of leukemic cells for about 40 days, following scarification.

No immunity could be induced when the scarification was performed with leukemic cell extracts inactivated by heatineg to 56°C for 1/2 hour.

The remarkable ability of the skin to activate a specific, defensive mechanism against leukemia, following intradermal or epidermal inoculation of small doses of leukemic cell extracts, is of considerable importance. Further, more detailed studies are now in progress.

The induced immunity appears to be durable. We have recently tested some of our guinea pigs which had been immunized in our earlier studies by intradermal inoculation of small doses of leukemic cells [3] and we have observed that the induced immunity is still fully active after 2 years [unpubl. data].

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In addition, 5 scarified guinea pigs received an intradermal challenging reinoculation (0.2 ml each) of a leukemic cell suspension of 10^{-3} concentration, and 3 developed leukemia (table II). In a control group, 5 untreated guinea pigs were inoculated intradermally with the same dose of leukemic cells, and all developed leukemia.

Microscopic studies, which are now in progress, will be reported in a separate communication. Preliminary studies revealed that after 2 weeks, following the scarification, leukemic cells, containing many virus particles similar to those previously described [2] could be found in strands and clusters, infiltrating the superficial layers of the dermis. The leukemic cells gradually disintegrated and disappeared within 4–5 weeks after scarification.

No resistance was induced with heated (56°C, ½ hour) leukemic extracts. Five guinea pigs had their skin scarified with heated (56°C, ½ hour) leukemic extracts. After 30 days, the 5 guinea pigs were reinoculated subcutaneously (0.5 ml each) with a leukemic cell suspension of 10^{-2} concentration. All 5 developed leukemia within 2–3 weeks after inoculation.

Discussion

We have demonstrated in our previous studies [3, 4] that it is possible to immunize guinea pigs of a susceptible strain against the inoculation of L2C leukemia by intradermal inoculation of very small doses of leukemic cell suspensions. Following development, and subsequent regression, of small intradermal tumors resulting from such inoculations, the animals are in most instances immune to reinoculation of heavy doses of leukemic cell suspensions by any route [3]. Females are more resistant than males; they can be more readily immunized and once immunized, they can tolerate heavier challenging doses [4, unpubl. data]. The immunity, thus induced, could not be transmitted by serum collected from immunized animals [4].

Because of the relatively high mortality of guinea pigs inoculated with leukemic cells by the intradermal route [3, 4] we have now attempted to induce immunity by superficial scarification of the skin with leukemic cell suspensions. This procedure proved to be successful in inducing immunity in guinea pigs. The incidence of leukemia developing as a result of skin scarification with leukemic cell extracts was relatively low, provided that a suitable dilution (10^{-4}) of leukemic cells was employed and that the scarification was superficial and performed with sufficient care to avoid inadvertent puncturing of the skin.

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We have therefore attempted to establish whether the differences in RNA metabolism previously observed between single blast cells could be dependent on cell cycle. As the latter has been shown to be related to cell size, we have studied the relationship between cell size on one hand and the degree of uridine uptake as well as the rate of nuclear-cytoplasmic shift of label on the other hand. We have also investigated in each case the degree of thymidine incorporation in the different classes of cells, established on the basis of cell size measurements.

Materials and Methods

We have carried out autoradiographic investigations and cell size measurements with a Leitz micrometric ocular in one normal subject, whose lymphocytes were cultured *in vitro* with phytohaemagglutinin (PHA), in one haematologically normal subject, whose bone marrow was aspirated and examined, and in 8 cases of acute leukaemia, comprising different cytological varieties. In all these 8 cases, studies were performed on bone marrow cultures.

The *in vitro* culture and autoradiographic techniques were the same as in our previous investigations [7]. Samples from each culture were obtained after 1, 3, and 6 h.

Cultures were also set up using actinomycin D at a concentration of 10 µg/ml added 30 min before the radioactive precursor, in order to study its effect on the rate of RNA synthesis.

Blast cells were subdivided in the majority of cases into 4 different groups. In the first group were included the smaller blast cells ranging in size from 11 to 14 µm, in the second blast cells from 14 to 16 µm, in the third blast cells from 16 to 18 µm, and finally in the fourth blast cells from 18 µm upwards. Occasionally a slightly different subdivision was necessary because of the particular size distribution of the blast cell population.

Results

The results obtained in the PHA culture of normal human lymphocytes are reported in figure 1. Our data show that the degree of uridine incorporation and the extent of cytoplasmic labelling are directly related to cell size, inasmuch as the smaller cells are less heavily labelled than the larger PHA blast cells. However, in judging these results, it is necessary to remember that the process of PHA transformation entails an increase in cell size, which is paralleled and associated by a progressive enhancement in the rate of RNA synthesis. Therefore, in studying the transfer of uridine from nucleus to cytoplasm in PHA blast cells in relation to cell

Acta Haem 47 326–334 (1972)

Relationship Between RNA Metabolism and Cell Cycle in Acute Leukaemia

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Abstract Investigations were carried out in order to establish whether the differences in RNA metabolism previously observed amongst single leukaemic blast cells could depend on cell cycle While normal bone marrow precursors show a narrow range of variations in cell size and uridine incorporation in acute leukaemia differences are quite remarkable The larger blast cells characterized by a higher rate of thymidine incorporation show a more conspicuous degree of nuclear cytoplasmic shift of tritiated uridine compared to smaller blast cells The implications of these findings are discussed also in the light of recent biochemical findings

Key Words
Acute leukaemia
Autoradiography
Cell cycle in leukaemia
Leukaemia cells
RNA metabolism

In a previous paper [5] attention was drawn to differences in RNA metabolism between normal and leukaemic cells It was also pointed out [7] that within a given leukaemic cell population, single blast cells vary considerably with regard to the degree of uridine uptake and the rate of nuclear-cytoplasmic shift of label after a 6-hour period of chase Cells with low uridine incorporation and decreased nuclear-cytoplasmic transfer also exhibited weak or absent nucleolar labelling a finding which was considered indicative of defective nucleolar function These findings seemed to warrant a further extension of our investigations in order to determine the reason for the existence of such differences

A number of authors [2 3, 4] have suggested that from the point of view of cell kinetics, leukaemic blast cell populations may possibly be composed of 2 functionally distinct fractions one group of cells capable of division, and another composed chiefly of smaller cells which are out of cycle either permanently or for long periods of time

We have therefore attempted to establish whether the differences in RNA metabolism previously observed between single blast cells could be dependent on cell cycle. As the latter has been shown to be related to cell size, we have studied the relationship between cell size on one hand and the degree of uridine uptake as well as the rate of nuclear-cytoplasmic shift of label on the other hand. We have also investigated in each case the degree of thymidine incorporation in the different classes of cells, established on the basis of cell size measurements.

Materials and Methods

We have carried out autoradiographic investigations and cell size measurements with a Leitz micrometric ocular in one normal subject, whose lymphocytes were cultured *in vitro* with phytohaemagglutinin (PHA), in one haematologically normal subject, whose bone marrow was aspirated and examined, and in 8 cases of acute leukaemia, comprising different cytological varieties. In all these 8 cases, studies were performed on bone marrow cultures.

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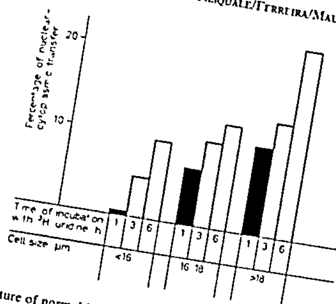


Fig 1 Culture of normal human lymphocytes incubated for 48 h with PHA

Table I Blast cells from normal human bone marrow incubated for 6 h with ³H uridine

Cell size, µm	Average nuclear grain count	Average cytoplasmic grain count	Percentage of nuclear-cytoplasmic transfer
< 14	86 66	27 83	24 30
14-16	77 14	24 85	24 36
>16	90 91	30 0	24 91

size, it must be borne in mind that most probably the smaller cells are 'intermediate' lymphocytes, which have not yet completed their transformation into fully developed blast cells and are therefore characterized by a less marked rate of RNA synthesis

Therefore, since PHA-blast cells do not belong to a metabolically homogeneous cell population, we have compared the rate of RNA synthesis of leukaemic cells with immature cells of a normal bone marrow, notwithstanding the considerable difficulties inherent in cell identification in autoradiographic preparations

Careful autoradiographic studies have shown that normal blast cells (myeloblasts as well as proerythroblasts) display a rather narrow range of

Table II Autoradiographic findings with ^{3}H-uridine and ^{3}H-thymidine in leukaemic cells and their relationship with cell size

Case No. Sex Age years	Type of leukaemia	Cell size µm	Percentage of labelling with ^{3}H thymidine after 1 h	Percentage of nuclear-cytoplasmic transfer of ^{3}H-uridine at different time intervals 1 h	3 h	6 h
1	monocytic	14	0	2.1	3.6	9.6
M		14-16	2	3.7	8	14.2
12		16-18	10	4.5	11.4	18
		18	22	6.9	13.4	20.4
2	myeloblastic	16	8	3.2	6.6	8
M		16-18	34	3.7	8.6	17.3
45		18-20	45	5.1	9.9	20.3
		20-22	50	7.3	10.5	23
		22	60	9	12.2	27.2
3	monocytic	16	15	0	5.69	6.87
F		16-18	22	0	8.15	10.01
37		18-20	30	0	11.25	14.91
		20	44	0	13.6	25.27
4	myeloblastic	14	0	0.6	6.49	14.43
M	promyelocytic	14-16	24	1.95	7.95	22.73
41		16-18	24	4.03	14.13	26.12
		18	54	5.91	14.22	34.23
5	lymphoblastic	14	1	1.29	9.35	11.73
M		14-16	6	3.25	16.1	18.35
14		16	32	4.73	15.99	22.51
6	myeloblastic	14	2	7.33	9.34	13.33
M		14-16	15	8.84	14.27	24.78
45		16	35	11.84	19.51	29.34
7	myeloblastic	14	0	0.93	4.03	10.45
F		14-16	4	2.13	7.32	14.45
31		16-18	14	3.02	10.55	19.11
		18	33	3.74	14.94	22.64
8	monocytic	16	8	0	6.19	14.63
F		16-18	22	3.74	8.07	17.95
50		18-20	32	5.44	10.95	22.31
		20	33	11.44	16.57	31.7

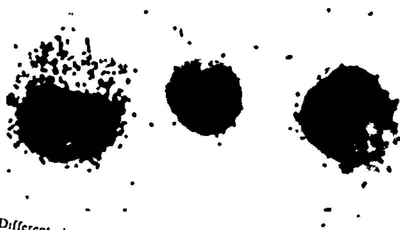


Fig 2 Different degrees of ^3H uridine incorporation and nuclear-cytoplasmic transfer in blast cells of varying size in a case of acute leukaemia

variations in cell size as well as of mean grain counts, as shown in table I Normal immature cells are fairly uniform with regard to cell size and rate of uridine incorporation In particular, all cells exhibited a marked shift of label from nucleus to cytoplasm after a 6 hour period of chase and most nucleoli were well labelled

The autoradiographic findings obtained in our 8 cases of acute leukaemia show that in leukaemic blast cells the picture is rather dissimilar The results are reported in table II, which, besides showing the differences in nuclear-cytoplasmic shift of label according to cell size, also gives an indication of the percentage of thymidine uptake in the various classes of cells

It may be seen that in all 8 cases of acute leukaemia cell size showed ample variations and each class of blast cells was characterized by a significant difference in the degree of uridine transfer from nucleus to cytoplasm

The difference in labelling intensity between the different classes was initially visible at the nuclear level but at the third and even more at the sixth hour the difference was most prominent also in the cytoplasm, the larger blast cells showed in fact a degree of nuclear-cytoplasmic transfer of label much greater than the smaller classes (fig 2) In the former, the degree of nuclear-cytoplasmic shift was practically similar to that of normal blast cells In 2 instances (cases 4 and 8) the percentage of cyto-

Table III Percentage of cells of various size at different time intervals

Case No	Cell size μm	1 h	3 h	6 h
1	14	45	23	6
	14-16	33	43	24
	16-18	17	25	25
	18	4	8	44
2	16	56	9	9
	16-18	28	45	21
	18-20	12	23	20
	20-22	1.33	20	38
	22	2.67	6	12
3	16	31	10	7
	16-18	35	28	43
	18-20	24	42	42
	20	10	20	8
4	14	38.67	50	34
	14-16	34	25	29
	16-18	*17.33*	*13*	13
	18	10	12	24
5	14	89	89	87
	14-16	10	9	9
	16	1	2	4
6	*14*	65	61	40
	14-16	29	33	47
	16	6	6	13
7	14	82	62	52
	14-16	8	20	30
	16-18	6	11	7
	18	4	7	11
8	16	89.5	66	82.5
	16-18	6	17	7.5
	18-20	3	14	5
	20	1.5	3	5

plasmic labelling after 6 h was even greater than that of normal immature bone marrow cells. It is of interest that in both cases leukaemic cells were in a rather advanced stage of maturation. Case 4 was a myeloblastic promyelocytic leukaemia, the blast cells of which showed numerous azurophilic granulations, rich in Sudan Black B and peroxidase positivity. Case 8 was a fairly well differentiated leukaemia, the blast cells of which

showed clear-cut monocytoid features and were characterized cytochemically by moderately coarse PAS positivity, numerous scattered sudanophilic granules and strong non-specific esterase activity.

After treatment with actinomycin D, we have observed an overall reduction in labelling intensity in both the nucleus and cytoplasm of the majority of leukaemic blast cells. However, as we have reported a few years ago [5, 6], also in the present investigation we have observed a number of leukaemic cells, which still show heavy uridine incorporation despite prior treatment with actinomycin.

The results obtained with tritiated thymidine confirmed that the larger blast cells are endowed with greater DNA synthetic activity than the other groups of smaller cells.

The study of blast cells of different size, at the various time intervals of the culture (table III), showed that the percentage of larger blast cells is initially low, never exceeding 10%, whereas it increases as time progresses, a finding which may be ascribed to a variety of reasons, such as the different rate of survival of the larger blast cells, to a selective destruction and death of the smaller blast cells or perhaps to alterations in membrane permeability with consequent swelling of the cell.

Discussion

Our autoradiographic findings indicate that in acute leukaemia marked differences exist not only in DNA synthesis as previously reported, but also in RNA metabolism between cells 'in cycle' and 'out of cycle'. This has also been suggested by FOADI *et al.* [1], who in acute leukaemia of childhood observed rapid RNA synthesis, following labelling with ^{3}H-uridine, in 20–40% of leukaemic blast cells, the highest grain counts being over the largest cells. However, the leukaemic population also contained cells in which no RNA synthesis was detectable and it was the author's impression that cells in cycle had the highest rate of RNA synthesis.

If the degree of nuclear-cytoplasmic shift of tritiated uridine may be taken as an indication of ribosomal precursor maturation [7], it seems reasonable to suggest that in the larger blast cells ribosomal formation takes place in a practically normal fashion. We have previously postulated the existence of a direct correlation between the rate of ribosomal formation and the proliferative activity of leukaemic cells [7]. Our present data seem to confirm such an hypothesis since a normal DNA synthesis

may be seen only in those cells in which the degree of nuclear-cytoplasmic shift of the radioactive RNA precursor is similar to that seen in normal bone marrow blast cells

Our findings also seem to indicate that the variable degree of ribosomal formation, expressed at the cytological level by the rate of nuclear-cytoplasmic shift of label, may also be responsible for the different extent of differentiation reached in each particular case by the leukaemic blast cell population The association in 2 of our cases (4 and 6) of intense uridine labelling, both nuclear and cytoplasmic, and of cytochemical features, characteristic of a fairly advanced stage of differentiation, is highly suggestive of such an hypothesis.

The results obtained with actinomycin D indicate that in some blast cells there are RNA fractions which are not degraded by this compound and are probably not DNA-dependent These results, as we have already mentioned, confirm our earlier observations and are in agreement with the data reported by Storti and Torelli [8], although the interpretation of their significance is still uncertain

The evaluation of the number of large blast cells in the first hour of culture shows that these cells, which, as we have seen, are endowed with a normal transfer of uridine from nucleus to cytoplasm, are generally scanty, usually under 10%. This observation is in keeping with the fact, ascertained by biochemical studies [9] that a certain amount of ribosomal RNA continues to be produced by a given leukaemic cell population, despite the general overall reduction

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Acta Haem. 47: 335–343 (1972)

Erythrocytic Enzyme Activities in Hypothyroid Children[1]

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Abstract. Several erythrocytic enzymes have been studied in children with hypothyroidism during the initial time of therapy with a combination of thyroxine and triiodothyronine: glucose 6-phosphate dehydrogenase, 6-phosphoglycerate dehydrogenase, lactic dehydrogenase, hexokinase, pyruvate kinase, phosphoglycerate kinase, glutathione reductase and glutathione peroxydase. The mean activities of all enzymes were lower in untreated hypothyroid patients than the mean activities of a control group. During hormonal treatment, the enzyme activities increased nearly in all patients regardless of whether they were subnormal or in the normal range. This increase is due to an elevation of the erythropoiesis and represents a decrease of the mean age of the circulating red cells.

Key Words
Erythrocyte enzymes
Hypothyroidism
Erythropoiesis and thyroid hormones

Hypothyroidism is associated with a slow metabolic rate. Since metabolism is a result of the function of all enzyme systems in the body, one can assume that the activities of the enzymes are lowered in hypothyroidism. This has been shown for dehydrogenases in the liver by HUGGINS and YAO [13] and YOUNG [26]. Serum enzymes in patients with hypothyroidism are often found to be slightly elevated [5, 9]. Some studies have been performed in erythrocytic glucose 6-phosphate dehydrogenase (G-6-PDH) with different results: a low activity of the G-6-PDH was found by GORDON and GANDOLLIA [8] and ROOT *et al.* [18] in some patients with hypothyroidism, whereas PEARSON and DRYAN [17] could not confirm this in a small group of only 4 patients. No other erythrocytic enzymes have been studied.

Therefore, we studied the enzyme activities of G-6-PDH, 6-phosphoglycerate dehydrogenase (6-PGD), lactic dehydrogenase (LDH), hexoki-

[1] Dr. Charles Huggins to his 70th anniversary.
Supported by »Deutsche Forschungsgemeinschaft«.

nase (HK), pyruvate kinase (PK), phosphoglycerate kinase (PGK) glutathione reductase (GR) and glutathione peroxydase (GSH-P) in patients with hypothyroidism before and during treatment with thyroid hormone

Material and Methods

Erythrocytes of 12 patients with hypothyroidism and of 24 healthy children of the same age serving as control group were examined G 6 PDH was checked in 2 more hypothyroid patients The diagnosis of hypothyroidism was based upon clinical symptoms a low growth rate a retarded bone age a low protein bound iodine and T_3 test and a high cholesterol in serum All patients were subsequently treated with a combination of thyroxine and triiodothyronine (T_4 and T_3,5 I) with doses being optimal for achieving a euthyroid state [7] During the observation period clinical symptoms of hypothyroidism disappeared a catch up growth appeared cholesterol and PBI and T_3 test were in the normal range Enzyme studies were performed before and during therapy with an interval of 3 to 5 weeks

The erythrocytes were washed 3 times with saline 0 9% and the leucocytes were removed with the supernatant Haemolysis was achieved by adding an equal amount of distilled water to the concentrated erythrocytes followed by deep freezing and thawing The supernatant obtained after centrifugation was used for enzyme determinations after diluting it to a haemoglobin content of 0 5 g per 100 ml The activity of enzymes obtained by this way for 1 0 g Hb was converted to the activity for 10^{11} erythrocytes All results are given in μmole substrate turnover in 1 min (= 1 IU) Each single value is based on a double determination

The activity of G 6 PDH (EC 1 1 1 49)[2] LDH (EC 1 1 1 27)[2] and PK (EC 2 7 1 40)[2] was measured using the enzyme kits produced by Biochemica Boehringer Mannheim The methods followed the description by KORNBERG and HORECKER [14] WROBLEWSKI and LADUE [25] and GUTMANN and BERNT [11] respectively For measuring the activity of 6-PGD (EC 1 1 1 44)[2] the method of HORECKER and SMYRNIOTIS [12] was used and for PGK (EC 2 7 2 3)[2] the method of BISHOP *et al* [4] The activity of HK (EC 2 7 1 1)[2] was determined according to GRIGNANI and LÖHR [10] of GR (EC 1 6 4 2)[2] according to BEUTLER and YEH [3] and of GSH P (EC 1 11 1a)[2] according to PAGLIA and VALENTINE [16] All biochemical reagents were purchased from Boehringer Mannheim perhydrol PA (H_2O_2) from E Merck Darmstadt

The enzyme determinations were performed under a constant temperature of 25 C in cuvettes of 1 0 cm lightpath at 340 nm in a photometer Eppendorf

Results

The mean enzyme activities of the patients with untreated hypothyroidism were lower than the ones of the control group However enzyme

[2] Enzyme nomenclature Elsevier Amsterdam 1965

Table I Enzyme activities in erythrocytes (µmole substrate turnover per minute 10^{11} erythrocytes)

Enzyme	Controls (n = 24) mean ± SD	n	Patients with hypothyroidism			
			before treatment		during treatment	
			mean ± SD	below normal range	mean ± SD	above normal range
G-6-PDH	14.0 ± 2.0	14	13.0 ± 2.5	5	16.7 ± 2.4	8
LDH	183 ± 35	12	174 ± 31	3	209 ± 21	4
6-PGD	7.7 ± 0.8	5	7.1	1	7.9	0
PK	8.6 ± 1.7	12	7.9 ± 1.8	3	9.0 ± 1.6	3
HK	1.7 ± 0.3	5	1.5	1	1.7	0
PGK	297 ± 33	5	276	1	295	0
GR	10.6 ± 2.3	12	8.3 ± 1.5	4	9.5 ± 0.8	0
GSH P	190 ± 39	12	172 ± 25	3	192 ± 23	2

Table II Haematologic values of patients with hypothyroidism before and during treatment with thyroid hormones

	Before treatment	During treatment
Erythrocytes, mill/mm³	3.1 ± 0.8	3.5 ± 0.9
Haemoglobin g/100 ml	9.6 ± 2.1	10.8 ± 1.9
Reticulocytes ‰	3–6	35 ± 15

activities below normal were found only in a few patients (table I). G-6-PDH was more often subnormal than other enzymes. The differences between the enzyme activities in hypothyroid patients and the controls were more significant for G-6-PDH ($p < 0.05$), GR ($p = 0.03$), LDH ($p = 0.05$) than for PK ($p < 0.2$) and GSH P ($0.2 > p > 0.1$).

During treatment with thyroid hormone, the mean activities of all enzymes increased during the first weeks. The most significant increase was observed for the mean activity of G-6-PDH ($p = 0.02$) compared with LDH ($p = 0.02$), GR ($p = 0.03$) and GSH P ($0.1 > p > 0.05$). The least mean increments were observed in PK ($0.3 > p > 0.2$).

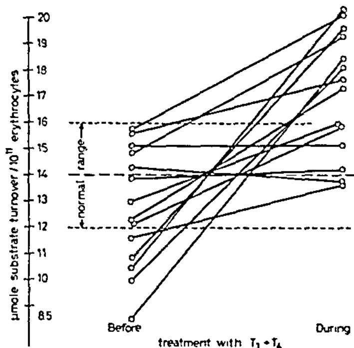


Fig 1. Activity of G-6-PDH in erythrocytes of children with hypothyroidism Before treatment with thyroid hormone the activity is often low but increases to high levels during treatment

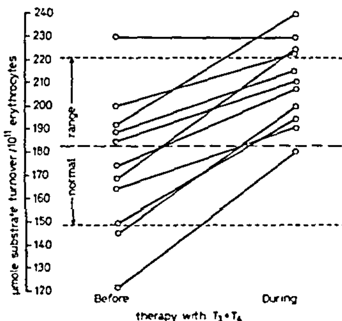


Fig 2 Activity of LDH in erythrocytes of children with hypothyroidism Before treatment with thyroid hormone enzyme levels are lower than during hormonal treatment

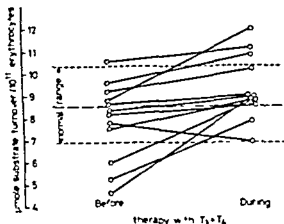


Fig. 3. Activity of PK in erythrocytes of children with hypothyroidism. An increase of the activity occurs when hormonal treatment is initiated.

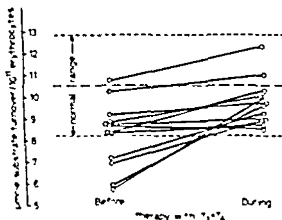


Fig. 4. Activity of GR in children suffering from hypothyroidism. Subnormal values increase to normal values during hormonal treatment.

The results for G-6-PDH, LDH, PK, GR and GSH-P are given in detail in figures 1 to 5. As documented, not in every single case an increase of the different enzyme activities was found. The highest rate of increments was present in G-6-PDH, reaching to values well above normal. In some patients also other erythrocyte enzymes showed activities above the range of the healthy controls.

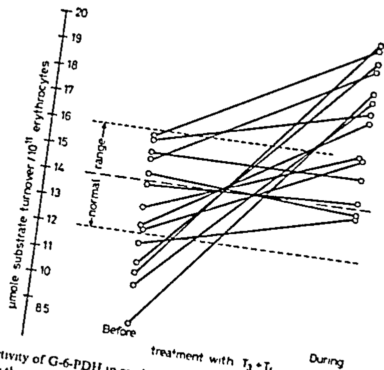


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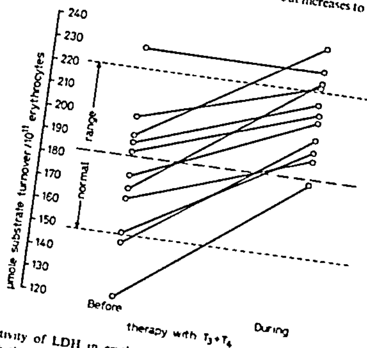


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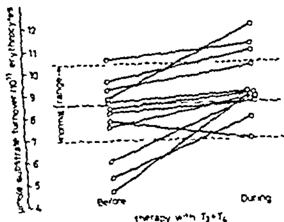


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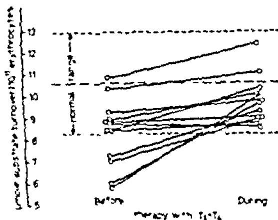


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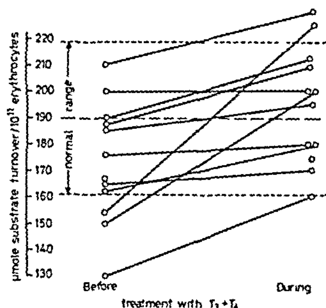


Fig 5 Activity of GSH P in children with hypothyroidism Subnormal values increase to normal values during treatment with thyroid hormone

During the observation period the erythrocyte count and the haemoglobin increased in patients with treated hypothyroidism (table II) An increase of the reticulocytes was also noted (fig 6)

Discussion

Thyroid hormone accelerates the intracellular metabolism by a direct influence on the oxydative phosphorylation [15], and an increased metabolism is based upon an elevation of enzyme activities Such elevation of the activity was found for different enzymes in liver cells of thyroidectomized rats after administration of thyroxine by HUGGINS and YAO [13] and YOUNG [26] The low activity of erythrocytic enzymes in a hypothyroid state and the normalization or further increase of the activity produced by thyroid hormones in our patients can be seen as the same process as in the liver cells In hyperthyroidism the activity of G-6-PDH is even elevated [2, 17]

Increments of enzyme activity in cells may be due to (a) a direct influence of the hormone itself on the enzyme molecule, (b) a decreased de-

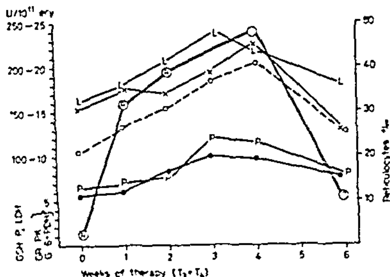


Fig. 6. Enzyme activities and reticulocytes during treatment of hypothyroidism. Within a short time after beginning hormonal therapy, reticulocytes and enzyme activities increase. When reticulocytes return to low counts, enzyme activities decrease.
R—R Reticulocytes, - - G-6-PDH, L—L LDH, P—P PK, ●—● GR, x—x GSH-P. (Values from one patient.)

struction or (c) leakage through the cell membranes and (d) a higher production of enzyme molecules.

An activation of the enzyme systems by thyroid hormone is very unlikely. Thyroid hormone added to red cells or to haemolysat *in vitro*, did not alter the enzyme activities, even not when we incubated the mixture for several hours at 37 °C before the enzyme assays were performed. Contrariwise WOLFF [23, 24] found a depression of the activity of isolated dehydrogenases by adding thyroxine.

The decrease of the enzyme activity after thyroidectomy in animals [13] or in hypothyroidism may be due to a higher destruction of the enzyme or a greater leakage through the cell membranes. The increased activity of serum enzymes in hypothyroidism is believed to result from changes in membrane permeability of muscle cells [5]. This point may be discussed also for erythrocytes which in hypothyroidism have a changed lipid concentration secondary to serum lipid disturbance, morphological-

ly, the erythrocytes present in an irregularly shaped form [21]. However, this gives not an explanation for the increase of enzyme activity in erythrocytes after treatment has been initiated.

The formation of new enzyme molecules seems to be the best explanation for the increased enzyme activity during hormonal treatment. However, no new protein can be produced in erythrocytes because of the lack of a nucleus. In liver cells, new enzyme production may be present.

Since no explanation can be given for the increase of erythrocytic enzymes on the base of the above-noted statements, another cause must be present. According to our observations that the red cell count and the haemoglobin content increased simultaneously during the initial time of treatment, we believe that the increase of the enzyme activities is due to a renewal of the erythrocytic population. The increase of enzyme activity goes parallel with the increase of reticulocytes as shown in figure 6. When the reticulocytes diminish, the enzyme activities return from high levels to normal values.

It is known that an aged red cell population has lower enzyme activities than a young red cell population, reticulocytes have even a higher specific enzyme activity than other red cells [1, 6, 19, 22].

During treatment of hypothyroidism, too, the increased enzyme activity documents a decreased mean age of erythrocytes. This phenomenon is secondary to erythropoiesis which is increased by thyroid hormones, as shown by SHIRAKURA *et al.* [20] in animal experiments. The increased erythropoiesis is believed to result from the increased metabolism and oxygen consumption.

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Acta Haem 47 344-347 (1972)

A New Method for Elution of Erythrocyte-Bound Antibody[1]

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Abstract A new method is described for eluting antibody from erythrocytes Sensitised erythrocytes are subjected to ultrasonic waves, elution probably depends on the application both of heat and great mechanical force It was successfully used to elute human and animal IgG and IgM antibodies and seed agglutinins By direct application of ultrasound to sensitised erythrocytes the time taken to produce strong eluates ranged from 30 sec for some seed agglutinins to 10 min for IgG anti D

Key Words
Antibody elution
Erythrocyte antibodies
Ultrasound

Elution of antibody from erythrocytes is an important procedure in blood group serology, chiefly in the investigation of autoimmune haemolytic anaemia [6] and haemolytic disease of the newborn [9] Several methods have been used with varying degrees of success [2, 3, 6] A particularly productive procedure is Rubin's modification [7] of the ether elution method of Vos and Kelsall [8]

It occurred to one of us (G W G B) that high-frequency acoustic waves might bring about elution of antibody, preliminary tests showed that this is true The idea is based on the fact [1] that the transportation of sound waves through liquid causes very rapid alternations of pressure so that large masses of minute bubbles of gas are formed The bubbles grow until a critical size is reached, when they implode causing shock waves, which produce great shearing stress

[1] Paper read at the First Meeting of the European Division of the International Society of Haematology Milan 10th Sept 1971

Table I Minimum time taken to produce strongly reacting eluates by the direct ultrasonic method

Type of agglutinins	Specificity	Type of sensitisation	Time, min
IgG	anti D	*In vivo* – haemolytic disease of the newborn	10
	anti-nl	*In vivo* – autoimmune haemolytic anaemia	10
	anti Kell	*in vitro*	2
	anti-Fy^{a}	*in vitro*	5
IgM	anti M (rabbit)	*in vitro*	5
	anti Le^{a}	*in vitro*	1
Seed agglutinins	anti A_1 (*Dolichos biflorus*)	*in vitro*	¼
	non specific (*Ricinus communis*)	*in vitro*	½
	non-specific (*Phaseolus vulgaris*)	*in vitro*	¼

Materials and Methods

Sensitised erythrocytes Erythrocytes were exposed to various antibodies under conditions appropriate to each, washed 4 times, and resuspended in an equal volume of physiological saline IgG and IgM antibodies and seed agglutinins were studied Erythrocytes sensitised *in vivo* were also studied They were obtained from a baby with haemolytic disease due to anti D and from a patient with autoimmune haemolytic anaemia due to anti nl

Exposure to ultrasonic waves The sensitised erythrocytes were exposed to ultrasonic waves generated by an MSE ultrasonic disintegrator (20 kc sec, 150 W, amplitude 12 μm peak to peak high power, transistor generator piezoelectric transducer)

Two methods were used In one the tube of washed sensitised cells was placed in a water bath and ultrasonic waves transmitted to the water In these conditions sound waves are transmitted through the glass tube to the cell suspension. In the other ultrasonic waves were applied directly to the cell suspension by means of a titanium probe No cooling device was used It is important that the tip of the probe should not be more than ¼ inch below the surface of the cell suspension.

Results

The indirect (bath) method is satisfactory for the cold acting IgM antibodies anti I and anti Pr, It is also satisfactory for eluting IgM anti-Le^{a} and for eluting agglutinins from the seeds of *Ricinus communis, Phaseolus vulgaris* and *Dolichos biflorus*

The direct (probe) method is more satisfactory but requires a very precise technique. The specificity of the various agglutinins studied and the minimum time taken to produce strongly reacting eluates is shown in table I.

Seed agglutinins which bind to single sugar molecules are understandably very easily eluted. Of the human and animal antibodies, IgG anti-Kell is more quickly recoverable in high concentration than other antibodies.

Discussion

It is doubtful whether any form of elution results in the recovery of an entirely intact antibody protein molecule. According to PIROFSKY [6] elution techniques which involve protein precipitation are particularly harsh, but heat elution for cold autoagglutinins is relatively harmless. Ultrasonic bombardment, besides generating heat, exerts great shearing force. It was terefore feared that the ultrasonic technique might be harsher than protein precipitation. Nevertheless we have so far obtained no evidence of denaturation and each agglutinin used to sensitise red cells retained its original specificity after ultrasonic elution. According to SVEDBERG, cited by DE KROMME and VERVAAT [4], ultrasound breaks up high molecular weight protein into subunits. The IgM antibodies which we eluted by ultrasound did not show any serological evidence of disruption. We do not claim that the ultrasonic technique is superior to others, by the probe method it is certainly quicker. Although the equipment is expensive, the method would be cheaper in the long run than protein precipitation methods.

During the investigation it came to our notice that MACPHERSON [5], in a similar application of this technique, liberated cell-bound vaccinia virus by ultrasonic vibration.

We should like to make it quite clear that this is only a preliminary report. We propose to study the ultrasonic elution method in greater detail before reaching any firm conclusions as to the advisability of applying it to routine blood group serology.

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Acta Haem 47 348–355 (1972)

Ineffective Erythropoiesis in Normal Rats and 'Early Labelled' Bile Pigment

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Abstract It has been shown using electron microscope autoradiography that the nuclei of maturing erythroblasts in the process of being extruded are labelled soon after the administration of radioactive iron This finding suggests that in normal animals at least part of the 'early labelling' of bile pigment, following administration of labelled haemoglobin precursors, could be due to the breakdown of the contents of the extruded nucleus during the normal process of red cell maturation rather than to the existence of ineffective erythropoiesis in which the entire cell is destroyed

Key Words
Autoradiography
Bile pigments
Electron microscopy
Erythroblast nucleus
Haeme synthesis
Ineffective erythropoiesis
Iron metabolism
Rat bone marrow

The major portion of the excreted bile pigment is derived from the breakdown of haemoglobin in senescent red blood cells Studies by LONDON *et al* [11] using ^{15}N-labelled haemoglobin precursors have shown that about 10% of the total excreted fraction in normal man can be recovered from faecal stercobilin after only 3 days Since this cannot have originated from the haemoglobin of senescent red cells it was suggested that it resulted from ineffective erythropoiesis, *i e* from the degradation of maturing red cells in the bone marrow, as is the case in many haemolytic disorders such as pernicious anaemia However, various other hypotheses have been put forward to explain this early labelling (a) turnover of myoglobin catalases, peroxidases or cytochrome enzymes, (b) production of haeme in excess of globin which is then converted into bile pigment (c) direct synthesis of pigment without involving the degra-

dation of a porphyrin ring, (d) turnover of non-haemoglobin haemes in the liver. No firm conclusion, however, has been reached as to the relative importance of these possibilities.

Many authors have demonstrated the presence of haemoglobin in the nuclei of erythroblasts using both staining methods with human erythroblasts [4] and microspectrophotometry with frog, chick and newt erythrocytes [5]. In non-mammalian vertebrates, haemoglobin can be recognised in the inter-chromosomal regions (euchromatin) of the nucleus as 'extremely fine textured material of low density, identical to the haemoglobin of the cytoplasm' [6]. This also applies in mammalian tissues and the euchromatin appears to be continuous with the cytoplam via the so-called nuclear pores.

It is fairly well accepted by many authors now that on maturation the nucleus of the erythroblast is extruded. Apart from electron microscope studies, BESSIS and BRICKA [2] have shown this using phase contrast microscopy combined with cinematography, and AWAI *et al.* [1] have observed that no reticulocytes contained label from 4 to 49 h after administration of tritiated thymidine. If a karyolytic process were taking place the reticulocytes early in their maturation phase should contain some DNA or degradation product of DNA.

This evidence for the presence of haemoglobin in the nucleus and the fact that the nucleus is extruded during maturation was not put forward as a source of bile pigment by LONDON *et al.* [11] or subsequent workers.

The results to be presented indicate that the haemoglobin present in the nucleus of erythroid cells is labelled soon after administration of a radio-active haeme precursor and as a consequence the process of nuclear extrusion during the normal maturation process will contribute to the early labelling observed in bile pigment.

Methods

F_1 hybrid rats of the inbred August and Marshall strains were injected intravenously with 5 [illegible] in sodium citrate (5 [illegible] body weight). Carrier free radioactive iron [illegible] was used to prevent saturation of the plasma iron binding capacity. After 6 h bone marrow from the femur was fixed in 3% glutaraldehyde, post fixed in 1% osmium tetroxide and embedded in Araldite. Autoradiographs were prepared using Ilford L4 emulsion, exposed 4–6 weeks and developed in [illegible] developer, mounted on grids and stained in alkaline lead hydroxide.

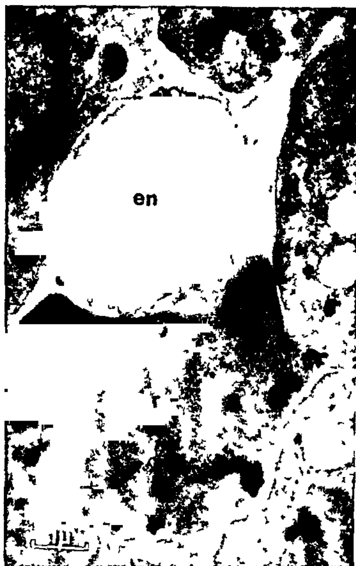


Fig. 1. Electron microscope autoradiograph of bone marrow 6 h after administration of ^{59}Fe showing labelled extruded nucleus (en) and reticulocyte (r) × 13,200

Results and Discussion

Figures 1 and 2 show labelled erythroblast nuclei in the process of being extruded 6 h after administration of radio-active iron. Figure 3 shows at lower magnification labelled erythroid cells at several stages of maturation.



Fig. 2. Electron microscope autoradiograph of bone marrow 6 h after administration of ^{59}Fe. Early erythroblast (ee), late erythroblast (le), extruded nucleus (en), reticulocyte (r). ×6,200.

A statistical analysis of the grain distribution in the bone marrow, together with a study of the resolution obtained with this isotope [12] has shown that the nucleus of erythroblasts contains on average 30% of the ^{59}Fe activity of the whole cell, and for more mature cells, just before extrusion, about 20%. This activity must be predominantly in haeme since by biochemical analysis we have shown that 90% of the activity in the marrow at this time is incorporated into haeme. Furthermore, in view of the conclusions of several workers – already referred to – that



Fig. 3. Electron microscope autoradiograph of bone marrow 6 h after administration of ^{59}Fe. Labelled extruded nucleus (en) still joined to reticulocyte (r). Early erythroblasts (ee) and late erythroblast (le). × 5 200.

the nucleus contains haemoglobin, it can be inferred that the labelling observed is in fact haemoglobin.

The observed nuclear labelling is probably due to a rapid entry into the nucleus of haemoglobin synthesised in the cytoplasm as suggested by KABAT [10] although it would also be consistent with the suggestion of HAMMEL and BESSMAN [7] that the synthesis occurs in the nucleus. Both



Fig. 4. Illustration of the so-called nuclear pores in erythroid cells. × 36,000

these studies were, however, with avian red blood cells. In view of the apparent existence of large nuclear pores (Fig. 4) the rapid transfer of label across the nuclear membrane seems likely.

The amount of activity lost on extrusion of the nucleus relative to the total activity resulting from the eventual breakdown of all the labeled erythroid cells depends on the activity incorporated into the nuclei, the

activity per cell and the relative number of nucleated compared to enucleated cells With radioactive iron the nucleated erythroid cells contain about the same activity per cell as the reticulocytes and are present in about the same numbers Since about 20% of the cells activity is in the nucleus the loss of activity on extrusion of the nucleus will be about 10% of the total activity incorporated into haeme

BESSIS *et al* [3] have suggested that some of the cytoplasm remains attached to the nucleus together with occasional mitochondria when it is extruded However this has not been observed in our preparations and we consider it unlikely that as much as 5–10% of the haemoglobin of the cytoplasm of orthochromatic cells is lost in this way with the nucleus as he claims

Although iron is reutilised after breakdown of haeme and so no ^{59}Fe activity will appear in the bile there will be excretion of activity from haeme labelled with ^{14}C or ^{15}N The distribution of these isotopes in erythroid cells will be the same as for ^{59}Fe and so one would expect about 10% of the activity excreted in the bile to be due to the extrusion of the nucleus This activity will appear in the early labelled fraction since the nuclei only contain label for 2–3 days after administration of the isotope all labelled cells by this time having matured and entered the circulation The early labelled fraction has been reported to be between 5–15% of the total activity appearing in the bile and could therefore be accounted for by the extrusion of the nucleus

ISRAELS *et al* [9] have demonstrated that early labelling of bile also occurs in ducks where the nuclei of erythroid cells are not extruded This indicates that other sources of labelled haeme exist but they do not report on the magnitude of this effect in ducks and furthermore this provides no information on the situation in mammals

In an earlier paper ISRAELS *et al* [8] report that the early labelling of bile also occurs in dogs where erythropoiesis has been arrested by administration of the cytotoxic agent Busulphan or by irradiation This also demonstrates that sources of bile pigment other than from haemoglobin exist but since an increase in early labelling was observed in dogs that had been bled there must also be a component associated with erythropoiesis Increased erythropoiesis will of course lead to an increase in the loss of haemoglobin by extrusion of the nuclei

Our observations suggest that the early labelling of bile pigment is in part due to the loss of haemoglobin on extrusion of the nucleus during the normal process of red cell maturation and together with those of Is

RAELS *et al.* [8] weaken the argument for the occurrence of ineffective erythropoiesis as a normal process in the maturation of erythroid cells.

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Acta Haem 47 356–360 (1972)

Vincristine-Induced Thrombocytosis Studied with ^{75}Se Selenomethionine

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Abstract Thrombocytosis was produced in rats by repeated doses of vincristine over a period of three weeks Using ^{75}Se Selenomethionine to assess platelet production, evidence was obtained that vincristine induces an increase in the platelet count by causing an increase in thrombopoiesis

Key Words
Platelet production
Selenomethionine
Thrombocytosis
Vincristine

Patients being treated with the Vinca alkaloids may show an unexpected increase in their platelet count [1, 3, 4, 10], and vincristine can also cause thrombocytosis in rats [10] How these myelotoxic drugs can induce an increase in the platelet count is not clear Studies in rats indicated that the thrombocytosis produced by vincristine did not result from a release of platelets from the splenic platelet pool [9] It has been suggested that platelet utilization may be decreased with a resulting increase in platelet life-span [11] On the other hand, SULTAN *et al* [12] treated a patient with vinblastine and found evidence that marrow production of platelets was increased

The present report describes the effect of repeated small doses of vincristine on the platelet count of rats and an assessment of the rate of platelet production in these animals using ^{75}Se Selenomethionine

Materials and Methods

Vincristine sulphate (Oncovin) was dissolved in normal saline each dose being contained in a volume of 0.4 ml and given by intraperitoneal injection The drug was stored at 4 C and its solution was discarded after 10 days storage Male albi

80 Wistar rats weighing 400 to 500 g were used. Blood samples were obtained from and intravenous injections given into a tail vein. Platelet counts were done by the method of BRECHER and CRONKITE [2].

Following an initial platelet count, 9 rats were given 0.04 mg/kg of vincristine by daily intraperitoneal injection. Doses were given on 6 days each week and the study lasted 3 weeks. Intraperitoneal injections of normal saline were given to 10 control animals. Platelet counts were taken from test and control animals on the 5th, 8th, 12th, 15th and 21st days of the study. To minimise the effect of bleeding on the platelet count, both the test and control animals were divided into 2 groups one group being counted on the 5th, 12th and 21st days, the other on the 8th and 15th days.

In the second study, the incorporation of ^{75}Se Selenomethionine into platelet precursors was used to assess the rate of platelet production. Five rats were given a 10-day-course of vincristine as described above, a similar number of animals being given saline. On the 10th day, ^{75}Se Selenomethionine, specific activity about 5 mc/mg, was injected into the tail vein of each animal in a dose of 50 μ. Blood samples were taken 48 and 96 h later, the platelets prepared for isotope counting and the percentage platelet utilisation of ^{75}Se Selenomethionine calculated as described by PENINGTON [8].

Results

The effect of vincristine, given in 6 daily doses each week, on the platelet count of rats is illustrated in figure 1. Counts taken on the 5th, 8th, 12th, 15th and 21st days of the study show that on each occasion there was an increase in the platelet count of the vincristine treated animals when this is related to their initial counts. The mean increase in the platelet count of the test animals throughout the study was 30%. In contrast, control animals receiving saline showed little change in their platelet count, the mean count decreasing by 6%.

In figure 2 is illustrated the percentage incorporation of ^{75}Se Selenomethionine into the platelets of vincristine- and saline treated animals, the isotope being given 10 days after the first dose of vincristine or saline. The results show an increased utilisation of ^{75}Se Selenomethionine in the vincristine treated rats at 48 and 96 h after its administration. At 96 h, the vincristine treated rats had, on the mean, 55% more of the isotope incorporated into their platelets than had the saline treated animals.

Discussion

Studies in rabbits and mice have shown that ^{75}Se Selenomethionine can be used to study the rate of platelet production [5, 8]. This isotope

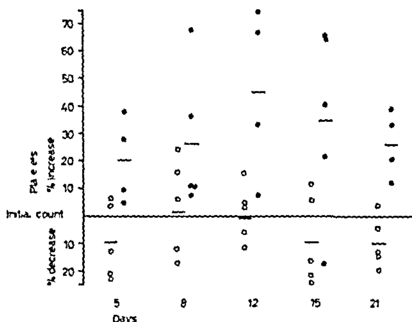


Fig 1 The effect of repeated daily doses of vincristine on the platelet count ○ Saline treated rats ● Vincristine-treated rats Horizontal bars indicate the *mean increase or decrease in the count*

is incorporated into proteins and is metabolised in a manner similar to methionine It is probably available for only a short time after injection during which it labels megakaryocytes and the proteins of their platelet precursors Adsorption on the preformed platelets is negligible and incorporation of the isotope into platelets can be used to assess the rate of platelet production [8] The finding in our study that more of the isotope was incorporated into the platelets of the vincristine treated rats than into those of the control animals indicates increased marrow thrombopoiesis following treatment with the alkaloid This result does not support the hypothesis that the Vinca alkaloids cause a thrombocytosis by damaging platelet function and so increasing their life span [11] Other evidence that the Periwinkle alkaloids can stimulate thrombopoiesis has been found experimentally in rats [9] and also by SULTAN *et al* [12] in a patient with idiopathic thrombocytopenic purpura treated with vinblastine with a resultant thrombocytosis In this patient there was no evidence of defective platelet function and indeed the properties of the platelets suggested that they were newly formed

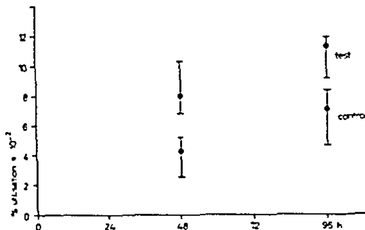


Fig. 2. Forty-eight and 96-hour platelet utilization of ^{75}Se Selenomethionine in vincristine treated (test) and control animals. Utilization is shown as the range and mean of results.

Vincristine is a myelotoxic drug and in appropriate dosage will cause marrow aplasia and thrombocytopenia in rats [9]. It is paradoxical that lower doses can apparently stimulate thrombopoiesis even when the drug is given for a prolonged period. Thrombocytosis may also follow treatment with another cytotoxic drug, methotrexate. However, this is transient, is preceded by thrombocytopenia and probably reflects an over compensation or rebound of the marrow to the initial depression of the platelet count [7]. Similarly in rabbits a single dose of vincristine can give rise to an initial erythroid hypoplasia of the marrow followed by a phase of hyperplasia [6]. The thrombocytosis induced by vincristine clearly results from a different mechanism. In our previous report, we found no evidence that thrombocytopenia preceded the thrombocytosis induced by the drug [9]. Further, in the present study thrombocytosis occurred during sustained dosage with vincristine and cannot be attributed to a rebound effect.

Acknowledgements. The work was supported by a grant from the Northern Ireland Hospitals Authority. We are grateful to the staff of the isotope department of the Belfast City Hospital for their assistance during the course of this study. We also wish to thank Miss P. Corry for her help in the preparation of the manuscript.

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Acta Haem 47 361–362 (1972)

Examination on a Sephadex Column of the Factor of Thrombocytosis-Inducing Effect in Normal Human and Mouse-Serum

A Preliminary Report

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Abstract From normal human and mouse sera β-globulin fractions were separated by electrophoresis and column chromatography The intraperitoneal administration of these fractions produced an increase of the thrombocytes in normal mice

Key Words
Column chromatography
Thrombopoietic factors

In previous investigations it has been established that the β-globulin fraction of normal human serum increases the thrombocyte count in mice being the most pronounced on the fifth day [1] The effect of this use is similar to that of the so-called active sera or from them obtained beta globulin fractions

Methods

The β-globulin separated by agar electrophoresis from normal human and mouse serum was chromatographed on a Sephadex G 75 column (The size of the column 60 · 1.5 cm, Na_2 EDTA tris buffer pH 8.2, flow rate 15 ml/h fractions [illegible] 5 ml/tube V_t = 250 ml The applied volume to the column was 4.5 ml the protein content was approximately 300 mg%) The extinction of the fractions was measured on a Spectromom 202 photometer at 280 nm in a quartz cuvette (thickness 1 cm) The peak obtained was lyophilized and then dissolved in physiological saline (fig 1)

Results

Immunologically the first peak contained mostly γ fractions. The second peak did not react with either antihuman or antimouse serum Its

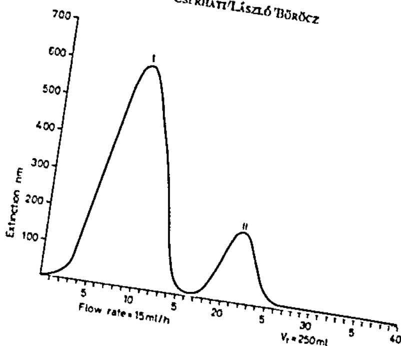


Fig 1

intraperitoneal administration increased the thrombocyte count of each animal significantly ($n = 14$, $t = 6\ 89$, $p < 0\ 001$) Similar results were obtained following chromatography of the β-fractions of normal mouse serum, as well from the β-fractions which had been separated after UV treatment [2] of the animals chromatographed on a Sephadex column

Concerning the nature of the peaks further investigations are in progress

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uthors address Dr I CSERHÁTI Dr I LÁSZLÓ and Dr A BÖRÖCZ, I Medinische Universitatsklinik Koranyi RKP 12, *Szeged* (Hungary)

E. ANTONINI and M. BRUNORI: Hemoglobin and Myoglobin in their Reactions with Ligands. North-Holland Research Monographs. Frontiers of Biology, vol. 21. North Holland Publ. Co., Amsterdam 1971. 436 pp., £ 11.05, US $ 28.–.

The new volume of the highly reputable series 'Frontiers of biology' is intended for investigators working in the field of heme protein research in chemistry, physical chemistry and biology. As pointed out by the authors in the preface, the aim of the monograph is to give a comprehensive account of the present knowledge on the reactions of hemoglobin and myoglobin with ligands, and of their interpretation in terms of molecular structure. The content is divided in the following 14 chapters: Preparation and some general properties of hemoglobin and myoglobin – The derivatives of ferrous hemoglobin and myoglobin – The derivatives of ferric hemoglobin and myoglobin – Structure of hemoglobin and myoglobin – Solution properties of myoglobin and hemoglobin – Ligand-dependent conformational changes – The equilibrium of hemoglobin and myoglobin with ligands – Kinetics of the reactions of hemoglobin and myoglobin with ligands – Specific aspects of the reactions of myoglobin with ligands – Specific aspects of the reactions of hemoglobin with ligands – Functional properties of modified hemoglobins and normal hemoglobin under special conditions – Oxidation reduction equilibria – Structure function relationships in hemoglobin and myoglobin – Models and theories of ligand binding in hemoglobin.

The book is written by competent scientists of high international reputation in hemoglobin and myoglobin research and will be an indispensable source of information and a standard reference work of interest to all investigators working in this field.

H. R. MARTI, *Aarau*

Varia

Deutscher Hämatologenkongress 1972

Der 18. Jahreskongress der Deutschen Gesellschaft für Hämatologie wird unter dem Vorsitz von *Prof. Dr. H. Martin*, Frankfurt, in der Zeit vom 1. bis 4. Oktober 1972 in Bad Nauheim stattfinden. Kongresshauptthema: Hämolyse. Anmeldungen sind zu richten an: *Zentrum der Inneren Medizin*, Abt. für Hämatologie, Theodor-Stern-Kai 7, 6 Frankfurt/M. (BRD).

Index rerum ad Vol. 47

Bearbeitet von G. Bothm, Basel

(B) = Book reviews – Buchbesprechungen – Livres nouveaux

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